National Library of Canada
Bibliothèque nationale du Canada

Canadian Theses Service
Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

NL-339 (r. 88/04) c

Canada

National Library of Canada
Canadian Theses Service
Ottawa Canada
K1A 0N4

Bibliothèque nationale du Canada
Service des thèses canadiennes

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-55371-5

Canada

THE UNIVERSITY OF ALBERTA

IN VITRO ADHERENCE OF *LACTOBACILLUS* SPP. TO RAT INTESTINAL CELLS

by

© TAMMY T. H. HOI

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

FOOD MICROBIOLOGY

DEPARTMENT OF FOOD SCIENCE

EDMONTON, ALBERTA

Fall, 1989

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR TAMMY T. H. HOI

TITLE OF THESIS IN VITRO ADHERENCE OF *LACTOBACILLUS* SPP. TO RAT INTESTINAL CELLS

DEGREE FOR WHICH THESIS WAS PRESENTED MASTER OF SCIENCE

YEAR THIS DEGREE GRANTED FALL 1989

Permission is hereby granted to the UNIVERSITY OF ALBERTA LIBRARY to reproduce single copies for private, scholarly or scientific research purposes only.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

(SIGNED) : Tammy T. H. Hoi .....

PERMANENT ADDRESS:

214-A, Yio Chu Kang Road

#02-20, Toho Garden

Singapore 1954

DATED August 18, 1989

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled

IN VITRO ADHERENCE OF *LACTOBACILLUS* SPP. TO RAT INTESTINAL CELLS

submitted by TAMMY T. H. HOI in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

FOOD MICROBIOLOGY.

Peter Sporns

Chairman

Michael E. Stiles

Supervisor

Susan Jensen

J. Ozimek

Date August 18, 1989

Dedicated to

my parents, my brothers and sisters,

and Gae.

ABSTRACT

Adherence of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus acidophilus* to intestinal epithelial cells was investigated in vitro using a tissue culture technique. Adult rat intestinal IEC-6 cells were cultured in tissue culture dishes to achieve a confluent monolayer. The monolayer of tissue cells was inoculated with bacterial suspension, incubated, and subsequently rinsed. Adherence was scored subjectively based on the presence or absence of bacterial cells after being stained with Giemsa stain.

Conditions such as growth medium, incubation temperature and stage of growth of bacterial cells, in addition to the influence of calcium ions on the ability of *Lactobacillus* spp. to adhere to rat intestinal (RI) cells were investigated. *Lactobacillus delbrueckii* subsp. *bulgaricus* strains J8 and J9 did not adhere to the RI cells under any of the conditions of growth and treatment. On the other hand, *Lactobacillus delbrueckii* subsp. *bulgaricus* strains J6, J10 and J11 and *Lactobacillus acidophilus* strain BG2-FO4 grown in MRS broth at 30, 37 and 40°C adhered well to RI cells, but not when they are grown in 10% reconstituted skimmilk (RSM). Strain J10 adhered well to RI cells during logarithmic and stationary phases of growth, indicating that stage of growth does not affect the adherence to RI cells. Calcium ions in bacterial suspension do not change the ability of bacterial cells to adhere to RI cells. This indicates that an adherence factor is present in *Lactobacillus* strains J6, J10, J11 and BG2-FO4 which enables them to adhere nonspecifically to RI cells. The ability of strain BG2-FO4 to adhere to rat intestinal cells in addition to human fetal intestinal cells in vitro demonstrates an apparent absence of host-specificity factor in this strain.

Studies of *Lactobacillus* strains were undertaken to investigate the genetic control mechanisms of adherence to RI cells. Using mini-scale plasmid isolation technique, it was shown that each strain contains one plasmid. Efforts to establish functions controlled by the plasmid failed to indicate a link with ability to adhere to RI cells. No relationship was established for antibiotic resistance, carbohydrate fermentation and bacteriocin production

traits with the plasmids. This study failed to identify a potential marker for screening plasmidless mutants. However, the results of this study indicate that adherence of *Lactobacillus delbrueckii* subsp. *bulgaricus* strains J6, J10 and J11, and *Lactobacillus acidophilus* strain BG2-FO4 to RI cells is chromosomally determined and the adherence factor remains stable at temperatures between 30-40°C throughout their growth cycle, but is suppressed by some factor(s) present in RSM.

There may be potential for developing a *Lactobacillus delbrueckii* subsp. *bulgaricus* strain that has favorable characteristics for use in the production of yogurt and also has the ability to adhere to human intestinal cells in order for it to deliver health benefits to the consumer.

Acknowledgements

I would like to express my gratitude to my supervisor, Dr. M. E. Stiles, for his guidance during the course of my Masters program. I should also like to thank M. Cerrone for her technical assistance in the laboratory and with the computer.

I am especially grateful to Dr. E. S. Jakobs for her guidance and assistance with the maintenance of tissue culture, which she generously provided for this work. In addition, I would like to express my appreciation for the use of facilities at J. S. McEachern Cancer Research Laboratory, University of Alberta.

I also wish to acknowledge all my friends and colleagues for their support and helpful discussions; and I especially appreciate my husband, Gae, who has been very understanding and supportive throughout this period.

Finally, I thank my sister, Rachel, for helping with the preparation of the typed manuscript.

This work was funded by Alberta Agriculture Farming for the Future Research Program and Alberta Dairy Industry.

TABLE OF CONTENTS

Chapter	Page
1. INTRODUCTION	1
2. LITERATURE REVIEW	4
2.1 Description of the genus *Lactobacillus*	4
2.2 Nutritional and Therapeutic Applications of *Lactobacillus* spp	5
2.2.1 Biosynthesis of B-Vitamins	5
2.2.2 Elaboration of Enzymes	5
2.2.3 Alleviation of Lactose Intolerance	6
2.2.4 Anticholesteremic Effect	7
2.2.5 Anticarcinogenic Effect	9
2.2.6 Antimicrobial Activity	11
2.2.7 Applications	13
2.3 Factors Affecting the Adherence of *Lactobacillus* species to Epithelial Cell Surfaces	15
2.4 Plasmid Mediated Traits of *Lactobacillus* spp	19
3. MATERIALS AND METHODS	23
3.1 Bacterial cultures	23
3.2 Maintenance of bacterial cultures	23
3.3 Bacterial culture media	24
3.4 Staining of bacterial cells	24
3.5 Tissue culture	24
3.6 Preparation of tissue culture medium	25
3.7 Growth and maintenance of tissue culture	25
3.8 Preparation of bacterial cells for adherence tests	26

3.9 Adherence test of *Lactobacillus* spp. to RI cells..... 27

3.10 Detection of antibiotic resistance..... 28

3.11 Methods for the detection of inhibitory substances produced by the adhering strains of *Lactobacillus* spp..... 30

3.12 Isolation of plasmids..... 31

3.13 Two dimensional gel electrophoresis..... 33

3.14 Estimation of plasmid size..... 33

3.15 Methods of plasmid curing..... 34

3.16 Screening for mutants..... 35

3.17 Tests on mutants..... 35

3.17.1 Carbohydrate fermentation..... 35

3.17.2 Adherence test of mutant strains..... 36

4. RESULTS..... 37

4.1 Cellular morphology of *Lactobacillus bulgaricus*..... 37

4.2 Adherence of *Lactobacillus* spp. to rat intestinal cells in vitro..... 37

4.2.1 Bacterial numbers at standard turbidity..... 37

4.2.2 Influence of cations on adherence of *Lactobacillus* strains to rat intestinal cells in vitro..... 37

4.2.3 Influence of growth medium and incubation temperature on adherence of *Lactobacillus* cells to rat intestinal cells in vitro..... 41

4.2.4 Effect of growth phase of *L. bulgaricus* strains J8 and J10 on the adherence to RI cells..... 45

4.2.5 Effect of chemical pretreatment of RSM cultures of *L. bulgaricus* strains J8 and J9 on adherence to RI cells..... 45

4.3 Characterization of *Lactobacillus* strains to develop a marker system for identification of mutants 49
4.3.1 Antibiograms 49
4.3.2 Production of inhibitory substances 49
4.4 Plasmid profiles of *Lactobacillus* test strains 52
4.5 Generation of plasmidless mutants from plasmid curing 55
4.6 Selected characteristics of mutant strains 58
4.7 Adherence test of plasmidless mutants 58
5. DISCUSSION AND CONCLUSION 62
6. REFERENCES 69

LIST OF TABLES

Table		Page
1	Antibiotics used to determine antibiograms of *L. acidophilus* and *L. bulgaricus* strains	29
2	Number of bacterial cells per mL of EBSS adjusted to O.D. 0.85 at 650 nm (grown in MRS and RSM)	39
3	Influence of calcium ions on adherence of *Lactobacillus* spp. to rat intestinal cells in vitro	40
4A	The effect of growth medium and incubation temperature of *L. bulgaricus* strains J6 and J10 on adherence to rat intestinal cells in vitro	46
4B	The effect of growth medium and incubation temperature of *L. bulgaricus* strain J11 and *L. acidophilus* BG2-FO4 on adherence to rat intestinal cells in vitro	47
5	Highest concentration of antibiotics (µg/mL) in doubling concentrations up to 256 µg/mL tolerated by *Lactobacillus* spp	50
6	Indirect screening for production of inhibitory substances by *Lactobacillus* spp. (Deferred Antagonism)	51
7	Characteristics of inhibitory substances produced by *Lactobacillus* spp	53
8	Adherence and inhibitory substance produced by cured strains of *L. bulgaricus* J11 and *L. acidophilus* BG2-FO4	59

LIST OF FIGURES

Figure		Page
1	Cellular morphology of *L. bulgaricus* strain J9 in MRS broth and in 10% RSM incubated at 37 and 40°C	38
2	Adherence of *L. bulgaricus* strain J10 to a confluent monolayer of RI cells in different suspending media with subsequent rinsing, using different rinse solutions	42
3A	Differences in adherence of *L. bulgaricus* strain J10 cells grown in MRS broth	43
3B	Difference in adherence of *L. acidophilus* BG2-FO4 cells grown in MRS broth	44
4	Growth curves of *L. bulgaricus* strains J8 and J10 in MRS broth at 37°C	48
5	Plasmid profiles of *Lactobacillus* strains J6, J8, J9, J10, J11, J11-HS3 and BG2-FO4, with *Escherichia coli* V517 as the standard	54
6	Migration of plasmids of *E. coli* V517 relative to molecular size	56
7	Plasmid profiles of *L. bulgaricus* strain J11 and its mutant strain J11-HS3, and *L. acidophilus* strain BG2-FO4 and its mutant strains FO4-1 and FO4-2	57
8A	The adherence of *L. bulgaricus* strain J11 and its mutant strain J11-HS3 to RI cells in vitro	60
8B	The adherence of *L. acidophilus* strain BG2-FO4 and its mutant strains FO4-1 and FO4-2 to RI cells in vitro	61

1. INTRODUCTION

Some species of *Lactobacillus* are inhabitants of the human gastrointestinal tract (Savage, 1979). These microorganisms play a role in maintaining the general health and disease states of the host through their metabolic functions. Elie Metchnikoff (Bibel, 1988) first attributed the concept of longevity of life to the consumption of "lactobacillic-fermented" dairy products. The proposed mechanism of action was by the replacement of certain 'undesirable' microorganisms in the intestinal tract by the more 'desirable' ones found in cultured milk products. Since then much research has been done to verify the proposed theory by establishing the health aspects and benefits associated with consumption of fermented dairy products.

Nutritionally, lactobacilli are associated with the B-vitamins (Shahani and Chandan, 1979) as these compounds are synthesized during microbial growth. In addition, enzymes such as lactase, proteases and lipases are elaborated as part of their metabolism (Chandan *et al.*, 1969a, b). These enzymes act by partially hydrolyzing their respective substrates i.e. lactose, proteins and fats in dairy products, thus increasing their digestibility. The ability to maintain a low cholesterol level, i.e. anticholesteremic effect (Mann, 1977a, b; Mann and Spoerry, 1974; Sinha, 1979), and the alleviation of lactose-intolerance in susceptible individuals (Gilliland and Kim, 1984) have been reported to be derived from the consumption of fermented dairy products. In addition fermented dairy products are also believed to have therapeutic effects for the frequent consumer. Anticarcinogenic activity has been associated with lactic cultures and fermented products. The anticarcinogenic activity is probably achieved by inhibition or degradation of carcinogens (Friend and Shahani, 1984a); by lowering the activities of procarcinogenic fecal enzymes (Ayebo *et al.*, 1980); or by suppressing tumor growth through stimulation of the immune system (Bogdanov *et al.*, 1962; Farmer *et al.*, 1975; Friend *et al.*, 1982; Kato *et al.*, 1983). The prevention and cure of certain intestinal disorders such as gastroenteritis, specific or nonspecific diarrhea, and

skin infections have all been documented as therapeutic effects associated with dairy products fermented with *Lactobacillus* spp. (Gordon *et al.*, 1957; Siver, 1961; Speck, 1980).

The beneficial effects are believed to be derived from the implantation or the establishment of a viable and stable population of lactobacilli in the gastrointestinal tract. It is essential therefore that the lactic acid bacteria survive passage into the gut as well as withstand the physiological environment of the gut. These organisms must overcome hostile factors such as gastric juice, bile acids, fatty acids, organic acids, hydrogen sulfide, lysozyme, antibiotics and the peristaltic movement of the gastrointestinal tract (Nieman, 1954). Several studies have shown that certain species of *Lactobacillus acidophilus* have successfully established themselves as a stable population in the gastrointestinal tract (Fuller, 1973; Watkins and Miller, 1983). The adherence of *Lactobacillus* spp. to the epithelial surfaces of the intestinal tract has been associated with polysaccharides (Fuller, 1975; Hood and Zottola, 1987) and lipoteichoic acids, secreted by these organisms (Sherman and Savage, 1986). In addition, Lin and Savage (1985) suggested that adherence is host-specific and is mediated by plasmids. In other studies, Conway *et.al.* (1987) found non-host-specific adherence in some *Lactobacillus* strains. However, Jonnson *et al.* (1985) demonstrated that some *Lactobacillus* strains isolated from an animal species could not necessarily survive and adhere to the gastrointestinal tract of the same test animal. At this point very little is known about the mechanism and mode of adherence of *Lactobacillus* to the gastrointestinal tract. *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) has not been studied extensively with regard to its adherence to intestinal cells. Studies in the early 1900's by several scientists (Herter and Kendall, 1908; Rahe, 1915) on *L. bulgaricus* demonstrated that these strains could not adhere and colonize the gut. Since then, *L. bulgaricus* has been neglected by most scientists as a potentially beneficial strain for the maintenance of health.

The object of this study was to investigate the in vitro adherence of some *L. bulgaricus* strains and a *L. acidophilus* strain to intestinal epithelial cell surfaces. The influence of cations, growth media, and incubation temperatures of bacterial cultures on the adherence was also tested. In addition, the genetic makeup of those adhering strains was determined in order to establish the mode of adherence genetically.

2. LITERATURE REVIEW

2.1 Description of the genus *Lactobacillus*

Members of the genus *Lactobacillus* vary in morphology from long slender rods to short coccobacilli which frequently form chains. They are Gram-positive, anaerobes or facultative anaerobes that are also nonspore forming and usually nonmotile. Some strains exhibit bipolar bodies, internal granulation, or a barred appearance when stained with Gram or methylene blue stain. *Lactobacillus* spp. are fermentative in their metabolism. The genus can be subdivided into three groups based on their fermentative patterns. The first group consists of the homofermenters, which produce more than 85% lactic acid from hexoses, without gas production. Pentoses and gluconate are not fermented by organisms in this group. *Lactobacillus acidophilus*, *L. delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*), and *L. helveticus* are typical members of this group. The second group consists of the facultative heterofermenters which produce about 50% lactic acid and considerable amounts of acetic acid. In addition, pentoses can be fermented. Some members of this group are *L. casei*, *L. plantarum*, and *L. sake*. The third group consists of the obligate heterofermenters that ferment hexoses to lactic acid, acetic acid, ethanol, and CO_2. Gas production is a characteristic of this group. Some members of this group are *L. bifermentans*, *L. brevis*, and *L. fermentum* (Schleifer, 1987). *Lactobacillus* spp. are aciduric and grow poorly at neutral pH or in alkaline conditions. Generally growth is best at pH 5.5 to 6.2 and continues beyond pH 5.0 down to as low as 3.6-4.0 depending on species. Their growth temperatures range from 2°C to 53°C with the optimum at 30-40°C. *Lactobacillus* spp. have complex nutritional requirements for amino acids, peptides, nucleotide bases, vitamins, minerals, fatty acids, and fermentable carbohydrates. Growth on solid media is often enhanced by anaerobic conditions in gas atmospheres containing 5-10% CO_2 (Kandler and Weiss, 1986).

2.2 Nutritional and Therapeutic Applications of *Lactobacillus* spp.

2.2.1 Biosynthesis of B-Vitamins

Many cultured milk products contain high levels of B-vitamins (Kwan, 1977; Moscoso *et al.*, 1973; Reddy, 1972; Reddy *et al.*, 1976; Reif *et al.*, 1976). The B-vitamin content is affected by the amount and type of lactic acid culture and the conditions of incubation (Hartman and Dryden, 1965). While many lactic acid bacteria require B-vitamins for growth, several lactic strains synthesize certain vitamins such as niacin, folic acid, and vitamins B6 and B12 (Reif *et al.*, 1976). The growth and end-products of these lactics are affected by growth media, temperature, and other environmental factors (Collins, 1977). According to Nilson *et al.* (1965), the biosynthesis of B-vitamins by these lactics is significantly influenced by temperature and length of incubation and other processing parameters.

2.2.2 Elaboration of Enzymes

It was first reported by Rasic *et al.* (1971) that the process of fermentation increases the biological value of proteins in yogurt. Similarly, many other researchers observed that yogurt can be digested more easily than milk (Breslaw and Kleyn, 1973; Simhaee and Keshavarz, 1974). It was proposed that proteases produced by the lactic cultures were responsible for the predigestion of milk proteins, thereby increasing the protein efficiency ratio (Hargrove and Alford, 1978, 1980). This was based on their observations that laboratory rats fed yogurt gained weight faster than those given unfermented or acidified milk. In addition, animals fed bulgaricus or acidophilus milk did not gain weight as fast as those fed yogurt. Reddy *et al.* (1976) reported that approximately 7% of the crude proteins in fermented cheese whey originated from cells of *Lactobacillus bulgaricus*. Furthermore, *Lactobacillus* spp. produced more free amino acids during fermentation than *Lactococcus* spp. (Amer and Lammerding, 1983). The enzymes elaborated during fermentation: protease; lipase; and lactase; degrade their respective substrates and predigest these

nutrients. The lipolytic and proteolytic activities of various lactic organisms were studied extensively by Chandan *et al.* (1969a, b). The specific lipolytic activities of lactobacilli were reported to be lower than those of lactococci, leuconostocs, and propionibacteria, whereas the proteolytic activities of lactobacilli were higher. *Lactobacillus bulgaricus* was reported to have a high proteolytic activity.

Casein is more readily hydrolyzed than whey proteins at pH 5.8-5.9. Poznanski *et al.* (1965) demonstrated the breakdown of casein using a combination of enzymes: proteinase from *L. bulgaricus* and peptidases from *Streptococcus thermophilus*. Bovine whey proteins, on the other hand, undergo a slow and irreversible aggregation in the presence of lactic cultures (Chandan *et al.*, 1970). These studies demonstrate the relative importance of lactobacilli in protein hydrolysis and of lactococci in lipid hydrolysis in fermented dairy products. In addition these factors contribute to the physical and nutritional properties of fermented dairy products (Shahani and Chandan, 1979).

2.2.3 Alleviation of Lactose Intolerance

The production of ß-galactosidase, also known as lactase, by lactic cultures during fermentation may be of significance to individuals who suffer lactose-intolerance. Goodenough and Kleyn (1976) observed a more efficient absorption of lactose in laboratory rats that were given yogurt containing viable cultures. The intestinal lactase activity of these test animals was considerably higher than controls fed other diets. The lactase enzyme is reported to be cell bound in *L. bulgaricus* and *S. thermophilus*. In vitro observations indicated that the digestion process enhanced the release of lactase (Kilara and Shahani, 1976). Gilliland and Kim (1984) observed an increase in lactase activity of yogurt cultures in the presence of bile salts. This indicates further the important function of these lactics in hydrolyzing lactose in the small intestine, where bile is present. In studies using human subjects, the hydrolysis of lactose was higher in people consuming yogurt containing viable organisms than those consuming yogurt containing non-viable lactic

organisms (Gilliland and Kim, 1984). Thus, viability of the organisms is of importance for the successful digestion of lactose in the human gut.

2.2.4 Anticholesteremic Effect

It was first observed that the Maasai (*sic.*) tribesmen of Africa have low serum cholesterol levels although their diets consist of large amounts of saturated fats and cholesterol from animal products. However, they also consumed large amounts of fermented milk (Biss *et al.*, 1971; Mann, 1977a, b; Mann and Spoerry, 1974). Such observations triggered investigations of the hypocholesteremic effect of fermented dairy products. The results from these studies were contradictory. While many researchers observed a lowering effect on cholesterol, others observed only a transient reduction or no significant effect on the lowering of serum cholesterol levels through the consumption of fermented dairy products.

The hypocholesteremic effect has been supported by several studies. Rao *et al.* (1981) and Grunewald (1982) observed a decrease in serum cholesterol level in rats fed fermented milk or milk containing viable *L. acidophilus*. Thakur and Jha (1981) also observed a significant decrease in serum cholesterol levels in laboratory rabbits fed diets supplemented with yogurt. Similar observations were reported by Kiyosawa *et al.* (1984). Other studies involving the use of laying hens also showed a lowering effect on the serum cholesterol level when they were fed viable cultures of *L. acidophilus* (Tortuero *et al.*, 1975). Similar effects were observed in newborn infants fed with a milk formula supplemented with *L. acidophilus* (Harrison and Peat, 1975). Hepner *et al.* (1979) confirmed the hypocholesteremic effect when they observed a significant decrease in the serum cholesterol levels of human volunteers consuming a diet supplemented with yogurt for one week.

On the contrary, Thompson *et al.* (1982) did not observe a significant difference in the serum cholesterol levels of human volunteers fed diets supplemented with unfermented

and fermented milks. In addition, Grunewald and Mitchell (1983) found no significant difference in the cholesterol levels of mice fed fermented acidophilus milk. The results of many other researchers did not support the therapeutic effect of fermented milk products (Massey, 1984; Pulusani and Rao, 1983; Rossouw *et al.*, 1981). Jasper *et al.* (1984) however, observed what they considered a transient effect of yogurt on serum cholesterol levels.

Such discrepancies may be due to host-strain specificity of lactobacilli (Grunewald, 1985). Other researchers suspect that the discrepancies are caused by differences in antimicrobial activity (Shahani *et al.*, 1977), and in lactase activity (Fisher *et al.*, 1985) of the different strains of lactic acid bacteria. Therefore, the hypocholesteremic effect of lactic cultures may only occur with specific bacterial strains in specific hosts. However, very little work has been undertaken to investigate in detail strain variations and their capacity to decrease serum cholesterol levels in humans.

It is widely reported that some strains of *L. acidophilus* are capable of assimilating different levels of cholesterol in vitro (Gilliland *et al.*, 1985; Grunewald, 1985; Jasper *et al.*, 1984). Gilliland *et al.* (1985) observed that the presence of oxgall enhances cholesterol assimilation under anaerobic conditions. This suggests that *L. acidophilus*, if present in the gut where bile is abundantly available, can be beneficial to the host by effectively reducing the serum cholesterol level.

Although the exact mechanism of the hypocholesteremic effect is not understood, one theory postulates the inhibition of the enzyme 3-hydroxyl-3-methyl glutaryl CoA reductase (HMG CoA reductase) by compounds present in fermented milk (Mann, 1977a). Other compounds such as orotic acid (Richardson, 1978), calcium (Mann, 1977b) and lactase (Howard, 1977) have been proposed as playing a role in the reduction of serum cholesterol levels. According to Speck (1976), some species of the genus *Lactobacillus* can break down bile acids causing an increase in neutral sterol excretion and bile acid turnover rate, thereby leading to a decrease of cholesterol in the serum.

2.2.5 Anticarcinogenic Effect

Numerous studies conducted over the past two decades indicated that fermented dairy products and lactic cultures possess anticarcinogenic activity. In general, the anticarcinogenic activity can be divided into three categories:

1) Elimination of procarcinogens,

2) Reduction of procarcinogenic fecal enzyme activity, and

3) Immunological suppression of tumor growth.

1) Elimination of procarcinogens

Nitrites can be converted to carcinogenic nitrosamines in the intestinal tract of humans. Dodds and Collins-Thompson (1984) found that some strains of *L. acidophilus* assimilate nitrite in vitro. Although the exact mechanism of nitrite uptake and degradation by these organisms is not fully known, the characterization of the nitrite reductase activity of *L. lactis* has been achieved (Dodds and Collins-Thompson, 1985). Hence, the presence of lactobacilli may reduce the incidence of carcinogenesis by decreasing the potential for the conversion of nitrites into carcinogenic nitrosamines.

2) Reduction of procarcinogenic fecal enzyme activity

Procarcinogenic compounds can be converted into carcinogenic compounds by fecal procarcinogenic enzymes such as azoreductase, ß-glucuronidase, and ß-glucosidase (Goldin *et al.*, 1980; Goldin and Gorbach, 1984). The activity of these enzymes has been correlated to the potential for carcinogenesis. Ayebo *et al.* (1980) observed a decrease in fecal ß-glucosidase and ß-glucuronidase activity in human subjects fed acidophilus milk as opposed to those fed milk. Sinha (1979) also observed a decrease in the activity of fecal ß-glucuronidase following the ingestion of viable *L. acidophilus*. Hawksworth *et al.* (1971) observed that ß-glucuronidase originated from the facultative and strictly anaerobic strains of Enterobacteriaceae, *Lactococcus, Lactobacillus, Bifidobacterium, Clostridium* and

Bacteroides. In contrast, Kent *et al.* (1972) only detected this enzyme in strict anaerobes, such as the *Peptostreptococcus, Corynebacterium, Propionibacterium, Bacteroides, Clostridium* and *Cantenabacterium*. Gadelle *et al.* (1985) reported the involvement of three genera, *Clostridium, Peptostreptococcus* and *Staphylococcus* in the generation of ß-glucosidase. These undesirable organisms can be replaced by viable cells of *L. acidophilus* thus favorably altering the microecology of the intestinal tract. In the presence of *L. acidophilus*, the levels, and hence the activities, of procarcinogenic fecal enzymes are low.

3) Immunological suppression of tumor growth

It has been suggested that tumor suppression is associated with different forms of lactic cultures. Kato *et al.* (1981) reported the effective use of whole lactobacilli cells, both dead and alive, in suppressing the growth of tumor cells. Bogdanov *et al.* (1975) reported the antitumor effect of the glycopeptide moiety of *L. bulgaricus*. Friend *et al.* (1982), using yogurt cultures, observed an antitumor effect associated with the insoluble fraction of sonicated cell mixtures of *L. bulgaricus* and *S. thermophilus*. However the soluble fraction was ineffective in tumor suppression. In addition, *Bifidobacterium infantis* was reported to have antitumor properties (Kohwi *et al.*, 1978, 1982). The cell wall fractions of *B. infantis*, but not its soluble constituents, successfully suppressed tumor growth in laboratory mice. These observations indicate that the tumor-suppressing trait was mediated by components present in the cell wall fractions of these bacterial strains.

Investigations of the mechanisms of tumor suppression by Ayebo *et al.* (1982) indicated that the antitumor effect was only detected in vivo but not in vitro. Based on this they suggested the involvement of the immune system. Several other findings suggest that tumor suppression is mediated through an immune response. Kato *et al.* (1983) observed that the intraperitoneal injection of *L. casei* significantly enhanced the phagocytic activity of peritoneal macrophages. The findings of Hashimoto *et al.* (1984) and Perdigon *et al.* (1986) further support the involvement of macrophages in tumor suppression.

2.2.6 Antimicrobial Activity

Dairy products fermented with lactobacilli have been reported to be beneficial to human health due to their ability to inhibit the growth of various common pathogens. Some species of *Lactobacillus* have been reported to exert antagonistic action against such organisms as enteropathogenic *Escherichia coli, Salmonella typhimurium, Clostridium perfringens* and *Staphylococcus aureus* (Shahani *et al.*, 1977; Vincent *et al.*, 1959). In associative growth studies, Gilliland and Speck (1977) demonstrated the inhibition of growth of foodborne pathogens such as *E. coli* and *S. typhimurium* by *L. acidophilus*. *L. casei* was shown to inhibit certain *Salmonella* spp., *Vibrio* spp., *E. coli*, and *S. aureus* (Yakult Honsha Co. Ltd., 1971), and *L. bifidus* was shown to exert a similar effect against *E. coli, Shigella* spp. and *S. typhimurium* in vitro (Poupard *et al.*, 1973).

The mechanisms and the exact nature of inhibition in each case is not fully understood. However, there are numerous reports of the existence of naturally occurring antibiotic-like substances produced by lactic acid bacteria. Shahani *et al.* (1976, 1977) isolated acidophilin from *L. acidophilus* and bulgarican from *L. bulgaricus*. Both substances were exceedingly active against a wide range of pathogenic and nonpathogenic bacteria. The production of antibiotic-like substances varied with different *Lactobacillus* strains and with environmental conditions such as pH, temperature and growth medium. Other lactic organisms also produce natural antibiotic-like substances. *L. lactis* subsp. *lactis* produces nisin (Mattick and Hirsch, 1949), *L. brevis* produces lactobrevin (Kavasnikov and Sodenko, 1967), *L. helveticus* strain 481 produces a bacteriocin, helveticin J (Joerger and Klaenhammer, 1986), and strains of *L. acidophilus* produce acidolin (Mikolajcik and Hamdan, 1975), lactobacillin (Wheater *et al.*, 1952), lactocidin (Vincent *et al.*, 1959), lactacin B, and lactacin F (Barefoot and Klaenhammer, 1983). It has not been verified however, whether some of these substances produced by the different strains of *L. acidophilus* are the same even though they have been given different names

by different groups of scientists. Lactocidin was characterized as a bacteriocin-like inhibitor as the substance was found to be nonvolatile, remained active at neutral pH, and after catalase treatment. Both gram-positive and gram-negative organisms were inhibited by cultures of *L. acidophilus* that produced lactocidin (Vincent *et al.*, 1959). Purified lactacin B was a bacteriocin that inhibited closely related species of lactobacilli such as *L. leichmannii*, *L. bulgaricus*, *L. helveticus* and *L. lactis* (Barefoot and Klaenhammer, 1984). Barefoot and Klaenhammer (1983) also reported the production of a bacteriocin, lactacin F by *L. acidophilus* strain 88. Lactacin F exhibited antagonistic activity against *L. fermentum* 1750, *Streptococcus faecalis* 19433, in addition to those species inhibited by lactacin B.

Bacteriocins of *L. acidophilus* that have broad spectra of inhibition against both gram-positive and gram-negative bacteria have not been reported. Available literature suggests that a wide range of inhibition results from combined effects of antibiotic-like substances, bacteriocin, hydrogen peroxide (H_2O_2) and organic acids such as lactic and acetic acids. Dahiya and Speck (1968) observed the production of H_2O_2 by *L. bulgaricus* and *L. lactis* at levels high enough to inhibit the growth of *S. aureus*. Price and Lee (1970) reported the inhibition of *Pseudomonas* spp. by H_2O_2 produced by *Lactobacillus* spp. Collins and Aramaki (1980) also reported the production of H_2O_2 by several strains of *L. acidophilus* in milk at a level high enough to inhibit *S. aureus* . The amount of H_2O_2 produced varies between strains and can be enhanced with continuous agitation either during growth at 37°C or during storage at 4°C. Similarly, *L. plantarum*, *L. bulgaricus* and *Lactococcus* spp. produce enough H_2O_2 in refrigerated milk to retard growth of psychrotrophic bacteria (Gilliland and Speck, 1975). During fermentation the production of lactic acid lowers the pH of the medium, thus preventing the growth of pathogenic organisms. In addition, volatile acids produced by some *Lactobacillus* spp. have antimicrobial activity at low oxidation-reduction potential (Sandine, 1979).

Lactic acid exists in either of two forms: the levo-rotatory (L(+)) or the dextro-rotatory (D(-)) form. Their relative concentrations depend on the type of lactate

dehydrogenase produced by the bacterial cell (Tamine and Deeth, 1980). While L(+) lactic acid is normally completely hydrolyzed in the body, D(-) lactic acid is slowly used and excreted. Excess levels of the D(-) form can cause metabolic disturbances (Amer and Lammerding, 1983). Some strains of *L. casei* produce only D(-) lactic acid. Thus, from a nutritional standpoint, it is important not to select strains that produce high levels of D(-) lactic acid. Lactic acid has been associated with certain physiological benefits such as improving the digestibility of milk through precipitation of proteins, improving the absorption of calcium, phosphorus, and iron, stimulating the secretion of gastric juice, accelerating the onward movement of the stomach contents and contributing as an energy source (Rasic and Kurmann, 1978).

2.2.7 Applications

Twinning-McMath (1959) suggested the use of *L. acidophilus* to develop a stable protective intestinal microflora to help eliminate pathogenic microorganisms. Sandine *et al.* (1972) and Shahani and Chandan (1979) reported the effective use of *L. acidophilus* in eliminating enteric pathogens. Such findings have created considerable interest in the use of lactic acid bacteria as a prophylactic and therapeutic means of treating gastrointestinal disorders and other diseases.

The consumption of acidophilus products by human volunteers results in an increase in fecal lactobacilli and a decrease in fecal coliform bacteria, probably due to the favorable antagonism of lactic acid bacteria (Ayebo *et al.*, 1980; Prajapati *et al.*, 1986). Beck and Necheles (1961) reported the beneficial effects of using *L. acidophilus* in diarrheal and other intestinal disorders in humans. Watkins *et al.* (1982), and Watkins and Miller (1983) demonstrated the effectiveness of using *L. acidophilus* in eliminating pathogens such as *E. coli* in infected gnotobiotic chicks. Tomic-Karovic and Fanjek (1962) demonstrated the ability of *L. acidophilus* in milk to inhibit pathogenic *E. coli* in vitro. *L. acidophilus* was used successfully to treat twenty infants suffering from

enteropathogenic *E. coli* diarrhea. Zychowicz *et al.* (1974) and Alm (1983a) investigated the effect of *L. acidophilus* in acidophilus milk in treating children infected with *Salmonella* and *Shigella*. The consumption of acidophilus milk resulted in a decrease in the duration of the *Salmonella* carrier state, and a 66% recovery was achieved immediately after treatment in both the *Salmonella* and *Shigella* infections. The favorable effect of acidophilus milk on the carrier state of *Salmonella* was attributed to the antibiotic-like substances produced by *L. acidophilus*. It was also reported that long term administration of acidophilus milk helped eliminate all cases of dysentery.

Yogurt has been reported to have an antagonistic effect against the growth of pathogens such as enteropathogenic *E. coli, Vibrio* spp., *Salmonella*, *Shigella* and *Clostridium* in vitro (Rasic and Kurmann, 1978). Both lactic acid and antibiotic factors in yogurt may be involved in the inhibitory effects against the various enteropathogens. These factors produce favorable conditions for the proliferation of intestinal lactobacilli and discourage the growth of pathogens. Niv *et al.* (1963) succeeded in eliminating infantile diarrhea using yogurt as a dietary supplement. Alm (1983b) investigated the inhibitory effect of fermented milk products against *Salmonella* and *Shigella* in the presence of human gastric juice. In the 7-10 hour incubation period, in the presence of gastric juice, yogurt was effective in reducing the growth rate of both organisms. However, acidophilus milk was not as effective under similar conditions.

In contrast to the above findings, Pearce and Hamilton (1974) found no beneficial effect on the incidence of diarrheal diseases. Clements *et al.* (1981) administered Lactinex, a prophylactic preparation of *L. acidophilus* and *L. bulgaricus*, to humans before and after experimental challenge with enteropathogenic *E. coli*. They also found no effect in alleviating diarrhea. In further studies, Clements *et al.* (1983) reported that although both *L. acidophilus* and *L. bulgaricus* survived passage through the stomach and remained viable in the small intestine for 3-6 hours, these organisms did not prevent or alter the course of enteropathogenic *E. coli* in causing diarrhea in human volunteers.

Such controversies indicate the necessity to conduct further clinical studies using double blind treatments with viable cultures of host specific lactic acid bacteria to assess whether disease protection can be achieved through administration of these bacteria.

2.3 Factors Affecting the Adherence of *Lactobacillus* species to Epithelial Cell Surfaces

The mechanism whereby lactobacilli colonize their epithelial cell habitats is obscure (Savage, 1983; Suegara *et al.*, 1975). One factor important in the process of colonization is the capacity of the organisms to adhere to the epithelium (Savage, 1983). According to Suegara *et al.* (1975), the mechanism of association depends largely upon the surface structures of the bacteria and the host cells. Proteins or protein-containing substances were implicated as mediators of adherence of lactobacilli to the rat intestinal cells in vitro (Suegara *et al.*, 1975). This was supported by data generated through Conway's study of the adherence of lactobacilli to gastric epithelial cells in mice (Conway *et al.*, 1985). In contrast, Savage (1972) suggested that indigenous lactobacilli attached to the gastric epithelial cells of rodents by a substance on their surfaces that may be an acidic polysaccharide. Brooker and Fuller (1975) observed the presence of filamentous strands between the surfaces of lactobacilli and epithelial cells and between adjacent bacterial cells in preparations of gastric epithelial cells of chickens. The samples were stained by reuthenium red and examined by transmission electron microscopy. From their observations, they also suggested that an acidic polysaccharide was the substance responsible for the adherence of lactobacilli to crop cells. In addition, Suegara *et al.* (1975) suggested the involvement of chondroitin sulfate A and gastric mucin, because it was observed that their presence inhibited the adherence of a *Lactobacillus* strain.

Fuller (1975) reported that *Lactobacillus* strains that were able to attach to epithelial cells of the chicken crop have protein receptors, specifically concanavalin A receptors on the surface of the bacterial cells and may be partially responsible for adherence. Recently,

Hood and Zottola (1987, 1988) observed that an adhering *L. acidophilus* strain produces a layer exterior to the cell wall which is stained by reuthenium red. Another substance has been reported to mediate the adherence of lactobacilli. Sherman and Savage (1986) demonstrated that all of the *Lactobacillus* strains that exhibited adherence to murine gastric epithelial cells were found to contain lipoteichoic acid in their cell walls.

Adherence of *Lactobacillus* spp. to gastric epithelial cells is host-specific. Savage *et al.* (1968) reported that only lactobacilli isolated from mice could attach to the keratinized epithelial cells of germ-free mice. Fuller (1973) observed that the *Lactobacillus* strains isolated from the feces of chickens only adhered to the epithelial cells of the host. Other *Lactobacillus* strains isolated from mammals such as the pig could not adhere to chicken crop epithelial cells. Suegara *et al.* (1975) emphasized the existence of host-specificity in *Lactobacillus*. They observed that some lactobacilli from rats such as *L. fermenti* and *L. acidophilus* strains adhered to the nonsecreting epithelial cells of the rat stomach, but failed to adhere to the chicken crop epithelial cells. Similarly, *Lactobacillus* strains isolated from chicken feces could not adhere to the epithelial cells of the rat stomach but could adhere to the epithelial cells of chicken crop. None of the *Lactobacillus* strains isolated from humans and swine could adhere to the epithelial cells of the rat or the chicken. Fuller (1978) demonstrated that a *Lactobacillus* strain isolated from rat feces failed to colonize the crop epithelial cells of germ-free chickens.

In contrast, Kotarski and Savage (1979) discovered that *Lactobacillus* strains isolated from a mouse and a pig adhered well to the surfaces of both secreting and nonsecreting epithelial cells of the stomach of mice. The yeast *Torulopsis pintopesii* also adheres nonspecifically to surfaces of the gastrointestinal tract of mice in vitro (Suegara *et al.*, 1979). In the presence of lactobacilli, the yeast normally colonizes only the secreting gastric epithelial cells in mice (Savage, 1969). However, it could colonize both secreting and nonsecreting epithelial surfaces in germ-free mice. Thus, under appropriate conditions, both in vivo and in vitro, the organism can associate with a surface which it normally does

not colonize even when the epithelial surface differs drastically in structure. Such findings indicate that the capacity of an organism to adhere to epithelial surfaces does not in itself dictate the specificity of the organism to colonize epithelial cell surfaces. Undoubtedly, the capacity to adhere must preceed colonization. In addition to the capacity to bind specifically to a particular type of animal cell, the colonization of a particular surface may also be determined by environmental conditions (Kotarski and Savage, 1979). The local environment of the gastric epithelium provides undefined conditions of nutrient availability, ionic strength, pH, oxidation-reduction potential and oxygen tension. These conditions influence the expression of the surface properties of microorganisms and electrostatic charges of the surfaces of both bacterial and tissue cells (Harris and Mitchell, 1973; Marshall, 1976). In addition, conflicts between findings in these experiments could be attributed to differences in bacterial strains, in epithelial cells and most importantly in the models and techniques employed by different researchers.

Besides strain-specificity and chemical involvement, there are other factors that play a role in the adherence of lactobacilli to epithelial cell surfaces. After a microorganism is ingested, it must first pass through the hostile environment of the stomach to reach the intestine for adherence and colonization. The stomach has extreme conditions with pH ranging from 2 to 8. It has been documented that the low pH in the stomach due to the production of gastric hydrochloric acid plays a role in the destruction of microorganisms (Giannella *et al.*, 1972). The bactericidal effect is very evident below pH 2.5 (Maffei and Norbrega, 1975). Petterson *et al.* (1983) demonstrated that lactobacilli survive in the stomach and the intestinal tract of humans. *S. thermophilus* and *L. bulgaricus* ingested in yogurt were found to survive the stomach and intestinal passage and were recovered in the feces of test animals (Bianchi-Salvadori *et al.*, 1984). *L. acidophilus* survives in high numbers after incubation in gastric juice in vitro. Their survival in low pH gastric juice was enhanced by the addition of skimmilk powder. The milk solids provide a protective effect for microorganisms against high acidity (Conway *et al.*, 1987).

Fuller (1975) reported that a pH range of 4 to 8 had no effect on the adherence of lactobacilli to epithelial cells of the chicken crop. In addition, the adherence of *L. fermenti* to pig epithelial cells was reduced only slightly after exposure to pH 2 (Barrow *et al.*, 1980). Hood and Zottola (1988) demonstrated that *L. acidophilus* adhere equally well to human intestinal cells in vitro after suspension in low pH media. Their adherence was observed to be similar to the untreated cells even after exposure to pH 2, 3, and 4 for up to 5 hours. Hence, it appears that exposure to a low pH environment for 5 hours has no effect on the ability of *L. acidophilus* to adhere to the human intestinal cells in tissue culture. In previous studies by Hood and Zottola (1987), it was demonstrated that an adhering *L. acidophilus* strain produced an exterior layer which was identified as acidic polysaccharide. They also reported that this layer of polysaccharide was not affected by exposure to high acidity (Hood and Zottola, 1988).

Fuller (1975) studied the nature of the surface components mediating adherence of lactobacilli to epithelial cells. Using in vitro methods he found that several factors, such as contact time between bacterial and tissue cells, pH, age of bacterial cultures, growth temperature of bacterial cultures, and the energy source in growth media had little or no effect on the adherence of *Lactobacillus* strains to crop epithelial cells. Similarly, harsh treatments such as heating to 100°C for up to 10 min, treatment with EDTA or surface active compounds had no effect. Wheat germ lipase had no effect but proteolytic enzymes had a slight effect on adherence.

The adherence of *L. fermenti* was lost as the age of culture increased (Suegara *et al.*, 1975), implicating denaturation of the components mediating attachment with death of cells. They also observed that old bacterial strains which had been transferred many times since their isolation from host cells, lost their ability to adhere to the same host cells. However, freshly isolated strains adhere strongly. Ellen and Gibbons (1974) reported that *Streptococcus pyogenes* attached to epithelial cells in the highest numbers when harvested near the end of the logarithmic phase of growth. This was attributed to M-proteins

produced in large quantities during this period of growth. In contrast, several investigators reported that active metabolism in lactobacilli was not necessary to mediate adherence (Fuller, 1975; Barrow *et al.*, 1980). As mentioned earlier, Hood and Zottola (1988) reported that after exposure of bacterial cells to pH 2 for 5 hours, they adhere equally well to human intestinal cells in tissue culture. In fact, after 5 hours in acid, no viable cells were recovered, yet these bacterial cells retained their ability to adhere.

Kleeman and Klaenhammer (1982) studied the adherence of *Lactobacillus* spp. to human fetal intestinal cells in vitro. Using thirty-two strains of *Lactobacillus*, they demonstrated the existence of two mechanisms of adherence. One mechanism which is nonspecific requires calcium for adherence of all strains tested. It was postulated that calcium acts as an ionic bridge between the negatively charged surfaces of bacterial and epithelial cells (Brooker and Fuller, 1975; Costerton *et al.*, 1978). It has been widely reported that divalent and trivalent cations are involved in many adherence systems, such as the adherence of Kanagawa-positive *Vibrio parahaemolyticus* (Carruthers and Anderson, 1979) and in *Bordetella bronchioseptica* (Plotkin and Bemis, 1980). However, Curtis (1962) suggested that these cations function mainly as physical forces or chemical bindings and not as a specific adherence mechanism. The other system reported by Kleeman and Klaenhammer (1982) does not require cations for adherence and was present only in four of the thirty-two *Lactobacillus* strains tested. The adherence was not affected by alterations in colonial morphology, multiple broth transfers, or by cellular stress applied during culture concentration, frozen storage or lyophilization. These observations indicate that adherence determinants are highly stable although not widely distributed among *Lactobacillus* spp.

2.4 Plasmid Mediated Traits of *Lactobacillus* spp.

Plasmids are extrachromosomal, autonomously replicating, circular DNA molecules which exist independent of the chromosomal DNA (Day, 1982). Plasmids are found in most bacterial species, and under normal circumstances a particular plasmid can be lost

without affecting the survival of the cell. Plasmids may contain genes that are essential or confer a selective advantage to cells in certain environments (Kondo and McKay, 1985). It is characteristic of plasmids to be unstable and they may be lost with a concomitant loss of particular phenotypic traits encoded by that plasmid. The process of plasmid curing results in the loss of plasmid DNA.

The presence of plasmids in the genus *Lactobacillus* was first noted by Chassy *et al.* (1976). Since then many publications have confirmed that strains of *Lactobacillus* harbor multiple plasmids (Klaenhammer and Sutherland, 1980; Lin and Savage, 1985; Morelli *et al.*, 1983; Vescovo *et al.*, 1982). Klaenhammer (1984) published a general method for the isolation of plasmids in lactobacilli. While some plasmids are cryptic, there are others that are associated with identifiable phenotypic characteristics of the bacterial cell. Chassy *et al.* (1978) reported that plasmids isolated from strains of *L. casei* are associated with lactose fermentation. Smiley and Fryden (1978) reported that both N-acetyl-D-glucosamine fermentation and acid production traits in *L. helveticus* strains are determined by a specific plasmid. Morelli *et al.* (1986) observed that proteinase production in *L. helveticus* strains is encoded by the same plasmid that encodes for acid production. Several reports have indicated that antibiotic resistance is encoded by naturally occurring plasmids in strains of *Lactobacillus* (Ishiwa and Iwata, 1980; Morelli *et al.*, 1983; Vescovo *et al.*, 1982).

Only recently, bacteriocin production by *Lactobacillus* spp. has been associated with specific plasmids (Muriana and Klaenhammer, 1987) although earlier studies indicated that bacteriocin production by lactobacilli is chromosomally mediated (Barefoot and Klaenhammer, 1983; Joerger and Klaenhammer, 1986; McCormick and Savage, 1983). Muriana and Klaenhammer (1987) discovered lactacin F, a bacteriocin produced by *L. acidophilus* 88. Their experiments provided direct evidence for involvement of plasmid determinants for lactacin F production and host cell immunity. However, the production of helveticin J, a bacteriocin produced by *L. helveticus* 481 was found to be chromosomally

mediated (Joerger and Klaenhammer, 1986). Similarly, a plasmidless wild-type strain of *L. acidophilus* was found to produce bacteriocin, lactacin B, indicating that lactacin B production is chromosomally determined (Barefoot and Klaenhammer, 1983). Hence a particular phenotype that may be chromosomally determined in one *Lactobacillus* strain may be plasmid determined in another. Plasmid encoded determinants can serve as markers for identification of mutants that have lost that plasmid. In addition, they can serve as potential vectors in genetic engineering, since it can be ascertained that the trait they encode is expressed in the target host.

Plasmid curing is an essential procedure to determine if a particular trait is encoded by a particular plasmid in bacterial cells. By comparing differences in phenotypic characteristics between plasmid-containing cells and plasmid cured cells, plasmid encoded determinants can be identified.

While most plasmids are quite stable, there are some that undergo spontaneous segregation and deletion (Trevors, 1986). In order to increase the frequency of plasmid segregation, the use of curing agents or certain curing procedures can be employed. In many bacterial strains the use of curing agents has been unsuccessful since no standard protocols exist for all types of plasmids. The most commonly used curing agents are the intercalating dyes such as acriflavine, acridine orange and ethidium bromide. They act by preferentially inhibiting plasmid replication (Trevors, 1986). Carlton and Brown (1981) reported that the effective concentration of a particular curing agent varies considerably in the range of 100 to 1000 fold, being highly dependent upon bacterial species, curing agent efficiency, and the mode of action of the curing agent. Ethidium bromide and acridine will not cure plasmids larger than 250 MDa (Stanisich, 1984). Coumermycin and novobiocin are inhibitors of DNA gyrase (Gellert *et al.*, 1976). DNA gyrase catalyzes negative superhelical turns into double-stranded, closed, circular DNA (Izaki, 1981). Incubation temperatures of 5-7°C above optimal growth temperature can also be used or in combination with curing agents as curing methods. Vescovo *et al.* (1982) were successful

in using elevated temperatures of 46-49°C as a curing method for two *L. acidophilus* strains. They were also successful in curing the plasmids of several strains of *L. reuteri* and *L. acidophilus* by using ethidium bromide or acriflavine.

Since the time of Elie Metchnikoff until the late 1970s there was little or no interest in the prophylactic or therapeutic potential of the lactobacilli. During the 1980s there were many studies on adherence of lactobacilli to various epithelial cells and their hypocholesteremic and anticarcinogenic effects.

However, few, if any of these studies involved *L. bulgaricus* as the organism of choice. It seems that this organism is assumed to lack either the ability to adhere to epithelial cells of the intestinal tract or the ability to promote healthful results in consumers. The fields of molecular biology and genetic manipulation of microbial cells give rise to opportunities to develop these characteristics in strains of *L. bulgaricus*, retaining the desirable characteristics of this organism in yogurt culture while incorporating the healthful aspects associated with adherence to the intestinal epithelium. This study re-examines the ability of *L. bulgaricus* to adhere to intestinal cells in vitro.

3. MATERIALS AND METHODS

3.1 Bacterial cultures

A. The *Lactobacillus* spp. used in this study for adherence testing were obtained from different sources:

(i) *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) strains J6, J8, J9, J10 and J11 donated by Dr. J. Mostert (Research Institute for Animal and Dairy Science, Irene 1675, S. Africa);

(ii) *Lactobacillus acidophilus* strains BG2-FO4 and ADH donated by Dr. T. R. Klaenhammer (Department of Food Science, North Carolina State University, Raleigh, NC).

B. The indicator strains for identifying bacteriocinogenic strains included :

(i) *L. bulgaricus* ATCC 11842 and *L. acidophilus* ATCC 4963.

(ii) Lactic acid bacteria UAL 9 donated by Dr. B. G. Shaw (AFRC Institute of Food Research, Langford, Bristol, UK). UAL 15 *Leuconostoc mesenteroides* ATCC 23368, UAL 16 *Lactabacillus plantarum* ATCC 4008, UAL 17 *Lactobacillus viridescens* ATCC 12706 and UAL 19 *Pediococcus parvulus* ATCC 1937 are also used as indicator strains for screening bacteriocinogenic strains.

C. *Escherichia coli* V517 was used as the standard strain for the estimation of plasmid size (Macrina *et al.*, 1978).

3.2 Maintenance of bacterial cultures

All *Lactobacillus* cultures were grown overnight in MRS broth (Difco Laboratories, Detroit, MI), at 37°C and each was stored frozen (-70°C) in vials with 10% sterilized glycerol. Before use, the cultures were thawed at room temperature. A 1% inoculum was transferred aseptically into MRS broth and incubated at 37°C for 18-24 h with subsequent subculture in the same broth. The UAL indicator strains were stored in

cooked meat medium (Difco) at 4°C and subcultured every 3 months. Before use, the cultures were grown in APT broth (Difco) for 18-24 h at 25°C with subsequent subculture in the same broth. *E. coli* V517 was stored in cooked meat medium (Difco) at 4°C and subcultured every 3 months. Before use, a 1% inoculum was transferred into tryptic soy broth (Difco) and incubated for 18-24 h at 25°C, with a second subculture in the same broth.

3.3 Bacterial culture media

MRS broth and agar (Difco) were used to propagate the test strains of *L. bulgaricus* and *L. acidophilus*. The indicator strains of lactic acid bacteria were propagated in APT broth and agar (Difco).

3.4 Staining of bacterial cells

Lactobacillus cells growing in MRS broth were visualized by staining with gram stain (Difco). Those growing in 10% reconstituted skimmilk (RSM) were stained with 1% methylene blue (Fisher Scientific Co., Fair Lawn, NJ). Cells were viewed under oil immersion lens (Leitz Canada, Willowdale, ONT).

3.5 Tissue culture

Adult rat small intestinal epithelial (RI) cells, IEC-6 (ATCC CRL 1592) were donated by Dr. E. S. Jakobs (J. S. McEachern Cancer Research Laboratory, University of Alberta). The cells were used at passage levels 17-25. The tissue cells were cultured in a monolayer with a growth medium of Dulbecco's Modified Eagle Medium (D-MEM) (Gibco Laboratories, Grand Island, NY), supplemented with 10% Nu Serum (Collaborative Research Inc., Two Oak Park, Bedford, MA). Nu Serum was used as a substitute for fetal bovine serum (Dr. E. S. Jakobs, personal comunication).

3.6 Preparation of tissue culture medium

D-MEM (Gibco) was prepared according to manufacturer's instructions. Sodium bicarbonate ($NaHCO_3$) was added at a concentration of 3.7 g/L, the medium was adjusted to pH 7.2 with 1 N HCl and filter sterilized using a sterile filter system with a 0.45 µm nylon membrane (Corning Laboratory, NY). It was then supplemented with 10% Nu Serum (Collaborative Research) and 0.025 IU insulin/mL (Sigma Chemical Co., St. Louis, MO). The tissue culture medium was stored at 4°C and used within one month of preparation.

3.7 Growth and maintenance of tissue culture

RI cells were routinely cultured in 25 cm^2 disposable sterile tissue culture flasks (Corning) at an initial density of $1x10^5$ cells/flask. The cells were allowed to grow in tissue culture medium for one week at 37°C in 10% CO_2 to reach a confluent monolayer before use or transfer. Tissue culture medium was changed twice during that period. For use in adherence experiments, the monolayer of cells was first rinsed with an aliquot of sterile physiological saline (0.85% NaCl). Cells were detached from the flask by adding 0.8 mL of trypsin-EDTA (0.125% trypsin in 1.325 mM ethylenediaminetetraacetate) (Gibco). After 2-3 min incubation at 37°C in 10% CO_2, the detached cells were suspended in 10 mL growth medium (37°C), and mixed thoroughly by pipetting 20 times with a 10 mL pipette to break up the cell clumps. Cell numbers were determined with a ZBI Coulter counter (Coulter Electronics, Inc., Hialeah, Florida), at settings: Amplification 4; Threshold 10; Aperture current 4. After cell numbers were determined, dilutions were made with tissue culture medium supplemented with gentamycin (10 mg/mL) (Gibco) to give 50 µg gentamycin/mL of growth medium. A 2 mL aliquot of the desired dilution was transferred into 35 x 10 mm tissue culture dishes (Falcon Laboratories, Oxnard, CA). The cells were incubated at 37°C in 10% CO_2 until a confluent monolayer was achieved. The generation time of RI IEC-6 cells is 19-22 h (i.e. the time for the cells to double in numbers) during

the logarithmic phase of growth (Quaroni *et al.*, 1979). Before use in adherence tests, each dish was examined for confluency of growth under an inverted microscope (Leitz, Diavert model). Each dish contained approximately $5x10^5$ cells when confluency was reached. RI cells were stored frozen in sealed vials in liquid nitrogen tanks at different passage levels. To revive the cells, a frozen vial was carefully removed, thawed in a 37°C water bath, opened aseptically and suspended immediately in growth medium (37°C) in tissue culture flasks (Corning).

3.8 Preparation of bacterial cells for adherence tests

The *Lactobacillus* strains were subcultured at least twice in MRS broth before use. After incubation at 37°C for 18-24 h, the cells were harvested by centrifugation at 4500 x *g* for 10 min at room temperature. To determine the effect of growth phase on adherence, bacterial cells were harvested at approximately 12 h (logarithmic phase) and 24-28 h (stationary phase) according to the growth pattern of each strain. The bacterial cell pellet was washed three times in phosphate buffered saline (PBS) or Dulbecco 'A' (Oxoid Laboratories, England, UK) at pH 7.2. The washed pellet was suspended in 1-2 mL of PBS, and used to adjust 8-10 mL of suspending medium to O.D. 0.85 at 650 nm using a Spectronic 20 (Bausch & Lomb, W. Germany). The suspending medium was Earle's Balanced Salt Solution (EBSS) (Kleeman and Klaenhammer, 1982), composed of NaCl 6.8 g/L; KCl 0.4 g/L; $CaCl_2.2H_2O$ 0.25 g/L; $MgSO_4$ 0.10 g/L; $NaH_2PO_4.H_2O$ 0.141 g/L; and dextrose 1.0 g/L, adjusted to pH 7.4 with 5% $NaHCO_3$ solution (Oxoid). The solution was filter-sterilized through a 0.22 µm Nucleopore filter (Nucleopore Corporation, Pleasanton, CA), capped tightly, and stored at 4°C. The pH was checked before each use. The EBSS suspending medium was modified for use in this study by (i) preparation without $CaCl_2.2H_2O$, and (ii) preparation with an additional 3.6 mM $CaCl_2.2H_2O$.

The number of colony forming units (CFU)/mL of bacterial suspension for each bacterial strain adjusted to O.D. 0.85 at 650 nm was determined by making serial dilutions with 0.1% peptone water (Difco) and plated onto MRS agar plates. After anaerobic incubation at 37°C for 24 h, the bacterial colonies were counted in a Colony Counting Chamber (New Brunswick Scientific, Edison, NJ).

Lactobacillus cells grown in steam sterilized 10% reconstituted skimmilk for 18-20 h were adjusted to pH 6.9 with 10% NaOH and sodium citrate was added to 1% final concentration. The mixture was allowed to stand for 15-20 min to allow for solubilization of the milk proteins (Sandine and Elliker, 1970). The bacterial cells were harvested by centrifugation at 8000 x *g* for 15 min at 4°C. After washing three times with PBS, the bacterial cells were used for adherence testing. Controls were made up of bacterial cells growing in MRS broth and pretreated in the same way as the RSM cultures before harvesting the cells.

3.9 Adherence test of *Lactobacillus* spp. to RI cells

The monolayer of RI cells was rinsed three times with EBSS (37°C), and inoculated with 2 mL of the bacterial suspension in one of the suspending media. After incubation at 37°C for 15 min the bacterial suspension was removed by vacuum suction and the tissue cells were rinsed three times with EBSS (37°C) or with EDTA solution comprised of 0.85% NaCl and 0.1% EDTA.$2H_2O$. Care was taken during addition and removal of solutions from the tissue culture dishes to ensure that the RI cells were not detached from the bottom of the dishes. After rinsing, the RI cells were fixed with methanol, stained with Giemsa stain (Quaroni *et al.*, 1979) and viewed under a microscope using a 100x oil immersion lens (Leitz). Adherence was determined subjectively as positive or negative based on the presence or absence of bacterial cells on the tissue cells. All work involving tissue culture was done under aseptic conditions in a laminar flow cabinet (The Baker Co., Inc., Sanford, ME).

3.10 Detection of antibiotic resistance

A marker system was developed for the bacterial strains that tested positive in the adherence test. Antibiotic resistance of bacterial strains was tested for use as a possible marker. The antibiotics used are listed in Table 1 at doubling concentrations up to 256 µg/mL of MRS agar. Appropriate concentrations of antibiotics were added to 15 mL of molten MRS agar, mixed, and poured onto a petri dish. Bacterial cultures grown in MRS broth at 37°C for 18-24 h were inoculated onto these antibiotic media using a replicating inoculator (Cathra International Systems for the Microbiolologist, Diagnostic Equipment, Inc., St. Paul, MN). The inocula were allowed to dry and plates were incubated anaerobically at 37°C for 24-48 h. Each plate was examined for growth of bacterial cells.

For use as a marker for screening mutants, polymyxin B, sulfadiazine, sulfamethoxypyridazine, colimycin and nalidixic acid was used at 32 µg/mL concentration.

TABLE 1. Antibiotics used to determine antibiograms of *L. acidophilus* and *L. bulgaricus* strains.

ANTIBIOTICS	SOURCE	ANTIBIOTIC GROUP
Penicillin G	Sigma[1]	Beta-Lactam
Kanamycin	Terochem[2]	Aminoglycoside
Neomycin	Sigma[1]	"
Gentamycin	Gibco[3]	"
Streptomycin	Sigma[1]	"
Tetracycline	Sigma[1]	Tetracyclines
Lincomycin	Sigma[1]	MLS Group
Chloramphenicol	Sigma[1]	Phenicols
Rifamycin	Sigma[1]	Rifamycin
Polymyxin B	B M[4]	Peptides
Sulfadiazine	Sigma[1]	Sulfonamides
Sulfamethoxypyridazine	Sigma[1]	"
Colimycin	Sigma[1]	Other
Nystatin	Sigma[1]	"
Nalidixic acid	Sigma[1]	"

1 Sigma Chemical Co., St. Louis, MO.

2 Terochem Laboratories, Edmonton, ALTA.

3 Gibco Laboratories, Grand Island, NY.

4 Boehringer Mannheim Canada Ltd, Dorval, P.Q.

3.11 Methods for the detection of inhibitory substances produced by the adhering strains of *Lactobacillus* spp.

The production of inhibitory substances was tested by the following methods (Tagg *et al.*, 1976):

(i) Deferred antagonism

The test organisms were grown in MRS broth at 37°C for 18-20 h and an inoculum of each strain was transferred onto MRS agar plates using a replicating inoculator (Cathra) and dried in a laminar flow hood. The plates were incubated at 37°C for 24 h in an atmosphere containing 10% CO_2 and 90% N_2. Subsequently, the plates were overlayered with 8 mL of sterile MRS soft (0.75%) agar that had been inoculated with 80 µL of an indicator strain to give approximately 10^5 cells per mL soft agar. The indicator strains were grown in either APT or MRS broth at 25 or 37°C, depending on the bacterial strain used. The overlayered plates were incubated anaerobically at 25 or 37°C for 24 h. ATCC indicator strains were grown in MRS and incubated at 37°C while the UAL indicator strain was grown in APT and incubated anaerobically at 25°C. After incubation for 24 h, the indicator lawn was examined for zones of inhibition surrounding each test strain.

(ii) Detection of bacteriocin in broth supernatant

Producer strains grown in MRS broth at 37°C for 24 h were centrifuged at 6000 x *g* for 10 min at 4°C. The supernatant was collected and adjusted to pH 6.5 with 10 N NaOH. Half of the supernatant was sterilized with chloroform at approximately 10% concentration, mixed thoroughly and allowed to separate out. The other portion of supernatant was heated at 62°C for 15 min in a circulating water bath. Soft agar (8 mL) was inoculated with 80 µL of indicator strain and overlayered onto MRS plates. A 20 µL aliquot of pH adjusted, and sterilized supernatant was spotted onto the surface of the indicator overlayer as soon as it solidified. The supernatant was dried in a laminar flow cabinet and incubated under anaerobic conditions at 25 or 37°C for 24 h. The indicator lawn was checked for zones of inhibition surrounding each supernatant spot.

(iii) Test for bacteriophage

The reverse side inoculation technique was used for this test. Wells were cut into thick MRS agar with a sterilized 7 mm cork borer. The bottom of each well was sealed with one to two drops of molten MRS agar. The well was filled with 50 µL of supernatant that had been pH adjusted (6.5). The supernatant was allowed to diffuse into the agar and dried in the laminar flow hood. The agar was inverted into another petri dish and the reverse side of the agar was overlayered with an indicator lawn. Plates were incubated anaerobically at 37°C for 24 h and checked for zones of inhibition around the wells.

(iv).Confirmatory tests for bacteriocins

Aliquots of supernatants of producer strains that had been adjusted to pH 6.5 and sterilized with 10% chloroform were treated with catalase (Sigma) to give a final concentration of 100 U/mL of supernatant and incubated at 37°C for 1 h; with purified protease from *Streptomyces griseus* (Sigma) or Pronase^{R} (Sigma) to give a final concentration of 1 mg/mL. Their respective controls consisted of supernatants treated with heat-treated (100°C for 30 min) catalase, protease or Pronase^{R}. The treated supernatants and the controls were spotted onto a fresh indicator lawn, incubated and examined for inhibitory zones around each spot.

3.12 Isolation of plasmids

The *Lactobacillus* strains that tested positive for adherence to RI cells were subjected to plasmid isolation based on the method proposed by Anderson and McKay (1983) with some modifications. A 1.5 mL sample of an overnight culture was transferred into a 1.5 mL polypropylene Eppendorf centrifuge tube (Fisher) and centrifuged in a microcentrifuge (BHG Hermie National Labnet Co., Woodbridge, NJ) at low speed for 10 min. The pellet was resuspended in 380 µL of 6.7% sucrose (w/v) in 50 mM Tris, 1 mM EDTA, pH 8.0, and incubated at 37°C for 5-10 min. A 96.5 µL quantity of lysozyme solution (Sigma, 15 mg/mL of 25 mM Tris, pH 8.0) was added. After incubation at 37°C

for 5-10 min, 48.2 µL of 0.25 mM EDTA in 50 mM Tris pH 8.0 and 27.6 µL of 20% SDS (w/y) in 50 mM Tris, 20 mM EDTA pH 8.0 were added to the suspension, mixed, and incubated for 10-15 min at 37°C to complete lysis. The contents of the microcentrifuge tube was mixed on a vortex mixer (Fisher) at the highest setting for 30 s and the pH adjusted to 12.5 by adding 27.6 µL of freshly prepared 3 N NaOH solution. The sample was heated in a 65°C water bath for 20 min and allowed to cool to room temperature (approximately 15 min). A 49.6 µL quantity of 2 M Tris-HCl pH 7.0 was added and mixed by gentle inversion for about 3 min, followed by addition of 71.7 µL of 5 M NaCl. At this point 40 µL of protease (20 mg/mL) purified from *Stretomyces griseus* (Sigma) was also added and the suspension was further incubated at 37°C for 30-60 min. Deproteinization of the sample was done by adding 700 µL of 3% NaCl-saturated phenol (Fisher). The tube was shaken, allowed to stand for 5 min, and centrifuged to separate the layers. The upper aqueous layer was transferred into a second microcentrifuge tube and deproteinization was repeated with 400 µL of the same phenol. To facilitate separation, 300 µL of chloroform was added, and shaken prior to centrifugation for 5 min. After separation, the upper aqueous phase was transferred into a third tube, and extracted with 600 µL of chloroform: isoamyl alcohol (24:1 v/v). After thorough mixing, the sample was centrifuged for 5 min and the upper phase removed into a fourth tube. To this, 1 mL of cold (-20°C) 95% ethanol was added and held at -20°C for at least 1 h. The DNA pellet was obtained by centrifugation for 15 min and dried in the laminar flow hood for approximately 1 h. The DNA pellet was dissolved in 10 µL TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5), and 5 µL gel tracking dye (0.05% bromophenol blue, 60% glycerol in 50 mM sodium acetate) that was previously treated with RNAse. The sample was allowed to stand for about 1 h before separation by gel electrophoresis on 0.7% agarose (Agarose NA, Pharmacia) in TAE buffer (Tris base 4.84 g/L; glacial acetic acid 1.14 mL/L; EDTA 1.49 g/L), pH 8.0 in a Gel Electrophoresis Unit GNA-100 (Pharmacia Fine Chemicals, Sweden). Samples were loaded into the wells of the gel and subjected to a constant voltage of 60V, supplied from a

Electrophoresis Power Supply EPS 500/400 (Pharmacia). After 3 h, the gel was removed and stained with 0.1% ethidium bromide (Sigma) in TAE buffer for 15-20 min, and viewed under UV light at 300 nm from a Foto UV 300 DNA Transilluminator (Fotodyne Inc., New Berlin, WI).

The DNA of *Escherichia coli* V517 was extracted using the same procedure and was used as a standard for estimating the size of plasmids of test strains (Macrina *et al.*, 1978). A 1% inoculum of *E. coli* V517 was transferred into tryptic soy broth (Difco) and incubated at 25°C for 12-18 h before plasmid extraction.

3.13 Two dimensional gel electrophoresis

This is a procedure for distinguishing different forms of plasmid DNA (Hintermann *et al.*, 1981). After gel electrophoresis, bacterial strains with more than one plamid band were further treated to determine if the bands were derived from one or separate sources of plasmid DNA. Agarose gels with the entrapped bands of plasmid DNA were exposed to UV light by placing the gels 30 cm away from the UV source (Fotodyne) for 15-20 min. Subsequently, the gels were subjected to electrophoresis in a direction perpendicular to the first run. After running for about 3 h at 60V in TAE buffer, the gels were removed and stained with 0.1% ethidium bromide in TAE buffer for 15-20 min, and viewed under UV light.

3.14 Estimation of plasmid size

The size of plasmids from *Lactobacillus* spp. isolated in the agarose gels was estimated by running the plasmids with the DNA from *E. coli* V517. From the pictures the distance migrated by each plasmid band of the standard was measured and a logarithmic plot of relative mobility versus known plasmid size was done to estimate the size of unknown plasmids from *Lactobacillus* (Macrina *et al.*, 1978). The relative mobility is the

distance migrated by the plasmid band relative to the migration distance of the smallest plasmid of the *E. coli* V517 standard.

3.15 Methods of plasmid curing

The *Lactobacillus* spp. that contained one or more plasmids were subjected to plasmid curing, using different curing methods.

(i) Intercalating dyes

The curing agents, acriflavine (Sigma) and ethidium bromide (Sigma) were used. A preliminary test was done to determine the minimum inhibitory concentration (MIC) of each curing agent for each of the strains tested. The concentrations used ranged from 5 to 80 µL ethidium bromide or 5 to 10 µL acriflavine per mL of MRS broth. After the MIC has been established for each curing agent against each strain, another series of concentrations was prepared around the MIC level and inoculated with approximately 10^4 bacterial cells/mL. Tubes that exhibited growth were diluted with 0.1% peptone water (Difco) and plated onto MRS agar plates. The plates were incubated anaerobically for at least 48 h and colonies were screened for mutants using the methods outlined in Section 3.16.

(ii) Sodium-dodecyl Sulfate (SDS) and Novobiocin

A series of MRS tubes containing 0.002% SDS (BDH Chemicals, Toronto) was prepared with concentrations of novobiocin (Sigma) ranging from 0 to 50 µL/mL of MRS broth. Bacterial cells were inoculated at 0.1% level (10^5 cells/mL), and incubated at 37°C for 48-72 h. Tubes that exhibited positive growth were plated onto MRS plates, and colonies were screened for mutants by methods outlined in Section 3.16.

(iii) Elevated temperature in combination with Novobiocin

Bacterial strains inoculated at 0.1% level in MRS broth were incubated at 47-48°C for up to 72 h, and subcultured at least once into the same broth and further incubated for 48-72 h. In addition, MRS broth was supplemented with novobiocin at concentrations of 2, 4, 8, 16, and 32 µL/mL and incubated with bacterial strains (0.1% inoculum) under

similar conditions. The tubes that had good growth were plated onto MRS agar plates and incubated anaerobically at 42°C for 48-72 h. Colonies were screened for mutants by methods outlined in Section 3.16.

3.16 Screening for mutants

Wild type strains of J6, J10, J11 and BG2-FO4 were bacteriocinogenic (see results p. 51). Colonies growing on MRS agar plates from the curing experiments were subjected to different treatments to identify mutants. Bacterial colonies were transferred with sterile tooth-picks into MRS broth and incubated at 37°C for 24-48 h. Each bacterial culture was streaked onto an MRS agar plate to obtain a pure strain. The pure strain was transferred into MRS broth, grown for 24 h and screened for bacteriocin production and for antibiotic resistance as described in Sections 3.10 and 3.11(i), respectively. Plasmid profiling (Section 3.12) was also done.

3.17 Tests on mutants

3.17.1 Carbohydrate fermentation

Wild type and mutant strains of *L. bulgaricus* were tested for carbohydrate fermentation. MRS was made from Difco ingredients without sugar and sterilized by heating at 121°C for 20 min. To this medium, filter sterilized 10% sugar solutions were added to give 0.5% final carbohydrate concentration as well as 0.004% chlorophenol red indicator (Shaw and Harding, 1985). The carbohydrates used included fructose, glucose and lactose (Kandler and Weiss, 1986). A 1% inoculum of wild type and mutant strains was separately inoculated into MRS broth containing the different sugars. Tubes were incubated for 48-72 h at 37°C and checked for growth of the inoculum by development of a yellow color.

3.17.2 Adherence test of mutant strains

Wild type and mutant strains were subjected to adherence test in vitro on monolayers of RI cells according to the method described in Sections 3.7 and 3.8.

4. RESULTS

4.1 Cellular morphology of *Lactobacillus bulgaricus*

Cellular morphology was observed by gram staining of the *Lactobacillus* strains. Atypical variations in morphology were observed in strains J8 and J9 but not in strains J6, J10, J11 and BG2-FO4. In Figure 1 it can be seen that strain J9 grew as irregularly curved cells in MRS broth at 37°C and 40°C but not in 10% RSM at these incubation temperatures. This phenomenon was not observed in the other *Lactobacillus* strains: J6, J10, J11 and BG2-FO4 under the same growth conditions. These cells were observed to form filaments of rod shaped cells in MRS and RSM at 37 and 40°C.

4.2 Adherence of *Lactobacillus* spp. to rat intestinal cells in vitro

4.2.1 Bacterial numbers at standard turbidity

The test strains of *Lactobacillus* were grown in MRS and harvested after 18-20 h incubation at 37°C. The bacterial cells were suspended in Earle's Balanced Salt Solution (EBSS) adjusted to O.D. 0.85 at 650 nm and the viable count (CFU/mL of EBSS) was determined (Table 2). Counts ranged from 10^7 to 10^8 CFU/mL with generally good comparisons of counts between duplicates. Strains J8 and J9 in MRS and RSM produced slime which was not removed with washing. This may have contributed to the turbidity of the suspension accounting for the lower counts with these strains. There was a similar difference in bacterial numbers between strains J8 and J9 and other strains, when they were grown in RSM at 37°C for 18-20 h (Table 2).

4.2.2 Influence of cations on adherence of *Lactobacillus* strains to rat intestinal cells in vitro

From the in vitro adherence test it was found that *L. bulgaricus* strains J6, J10, J11 and *L. acidophilus* strain BG2-FO4 adhered to rat intestinal (RI) cells (Table 3). The

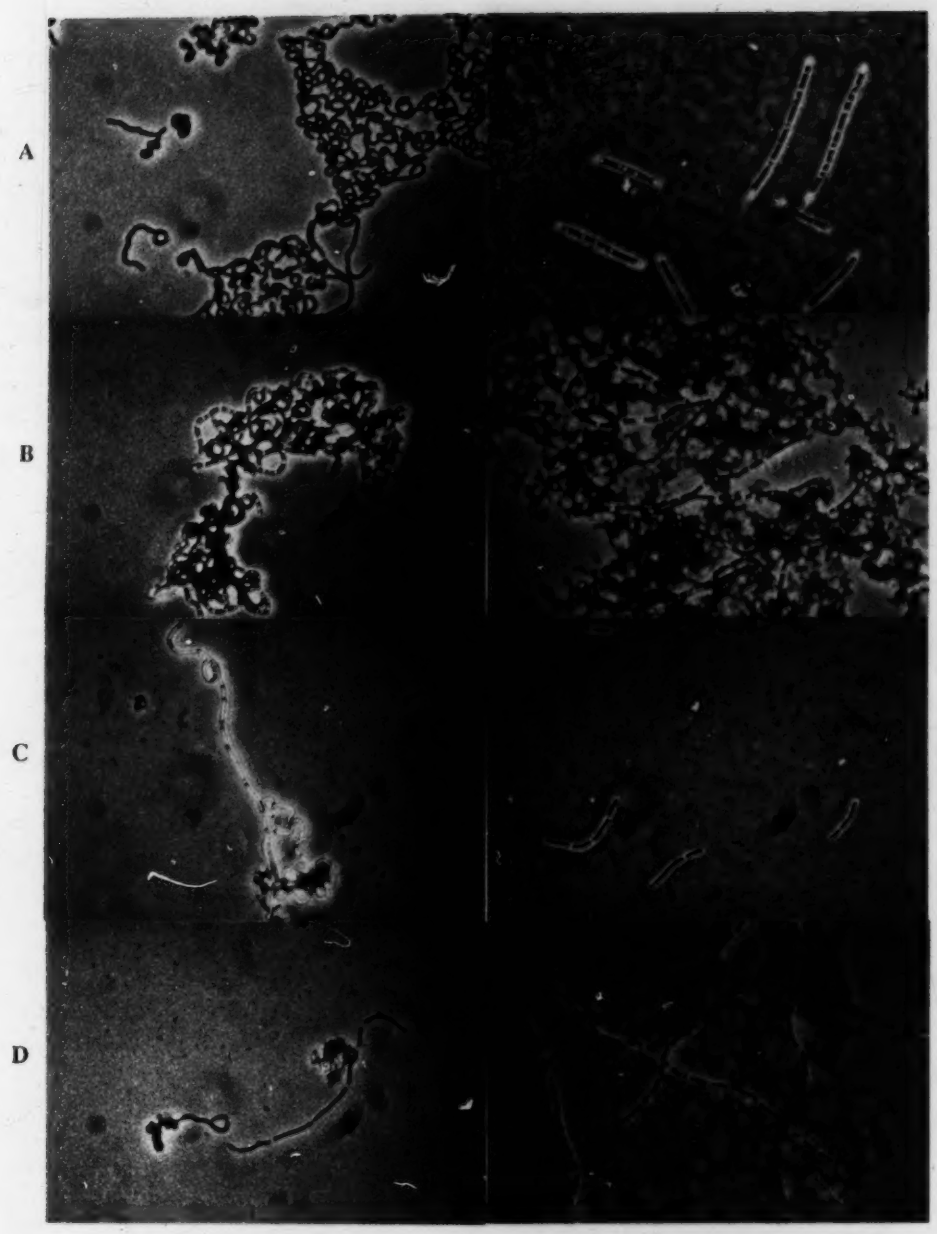


FIGURE 1. Cellular morphology of *L. bulgaricus* strain J9 in MRS broth and in 10% RSM incubated at 37 and 40°C.A) 6 h culture in MRS at 40°C; B) 24 h culture in MRS at 40°C; C) 10 h culture in MRS at 37°C; D) 24 h culture in MRS at 37°C; E) 6 h culture in RSM at 40°C; F) 24 h culture in RSM at 40°C; G) 6 h culture in RSM at 37°C; H) 24 h culture in RSM at 37°C This figure illustrates the variation in cellular morphology of strain J9. Curved cells are observed in MRS broth but not in RSM.

TABLE 2. Number of bacterial cells per mL of EBSS adjusted to O.D. 0.85 at 650 nm (grown in MRS and RSM).

Growth Medium[1]	*Lactobacillus* spp.	CFU/mL (duplicate determinations)	
MRS	J8	7.0×10^7	7.0×10^7
	J9	4.0×10^7	5.0×10^7
	J10	2.5×10^8	1.4×10^8
	J11	2.1×10^8	1.2×10^8
	BG2-FO4	3.5×10^8	3.5×10^8
RSM	J8	1.3×10^8	7.0×10^7
	J9	1.0×10^7	1.0×10^7
	J10	3.4×10^8	3.4×10^8
	J11	2.4×10^8	1.8×10^8
	BG2-FO4	NT[2]	

1. MRS = Lactobacilli MRS broth (Difco)
   RSM = Reconstituted (10% w/v) skimmilk powder
2. Not tested for this strain.

TABLE 3. Influence of calcium ions on adherence of *Lactobacillus* spp. to rat intestinal cells in vitro.

Lactobacillus spp.	Suspending Medium/ Rinse Solution			
	EBSS/EBSS[1]	EBSS/EDTA[2]	-Ca/EDTA[3]	+Ca/EBSS[4]
J6	+	+	+	+
J8	-	-	-	-
J9	-	-	-	-
J10	+	+	+	+
J11	+	+	+	+
BG2-FO4	+	+	+	+

+ = adherence; - = no adherence

1. EBSS suspending medium; EBSS rinse solution
2. EBSS suspending medium; EDTA rinse solution
3 EBSS minus calcium ions suspending medium; EDTA rinse solution
4. EBSS plus 3.6 mM calcium ions added to suspending medium; EBSS rinse solution

treatments involved suspending the bacterial cells in the presence of excess, or in the complete absence of calcium ions. In Figure 2A-C, adherence of *L. bulgaricus* J10 cells harvested from MRS broth, to a confluent monolayer of RI cells is illustrated. Adherence was observed with cells suspended in EBSS, EBSS with calcium ions removed, or in EBSS with addition of 3.6 mM calcium ions. The RI cells were subsequently rinsed with either EBSS or EDTA solution. No difference was observed in adherence of strain J10 under any of the conditions of treatment. Similar observations were made with *L. bulgaricus* strains J6, J11 and *L. acidophilus* strain BG2-FO4. However, it appeared that strains J10 and BG2-FO4 adhered more to RI cells than strains J6 and J11. This observation was based on the observation that bacterial cells of strains J10 and BG2-FO4 were more densely packed on the tissue cells. On the other hand, *L. bulgaricus* strains J8 and J9 did not adhere to RI cells under any conditions tested, as shown in Figure 2D and E. These observations indicate that calcium ions do not play a role in the adherence of *L. bulgaricus* strains J6, J8, J9, J10, J11 and *L. acidophilus* strain BG2-FO4 to RI cells.

4.2.3 Influence of growth medium and incubation temperature on adherence of *Lactobacillus* cells to rat intestinal cells in vitro

L. bulgaricus strains J8 and J9 were also tested for adherence after growth in 10% RSM at different temperatures for 18-24 h, following at least two subcultures in the same medium. Cells of strains J8 and J9 isolated from RSM did not adhere to RI cells, similar to cells grown in MRS at 30, 37, and 40°C. Differences in adherence for *L. bulgaricus* strain J10 and *L. acidophilus* strain BG2-FO4 grown in MRS broth or RSM is illustrated in Figure 3A and B, respectively. A marked decrease in the number of adhering cells is shown for *L. bulgaricus* strain J10 grown RSM, as compared to cells grown in MRS (Figure 3A). Whereas, for *L. acidophilus* adherence was almost totally eliminated by growth of the culture in RSM (Figure 3B). Cells of strains J6, J10 and J11 from overnight RSM cultures did not adhere to RI cells as well as those grown in MRS. The subjective

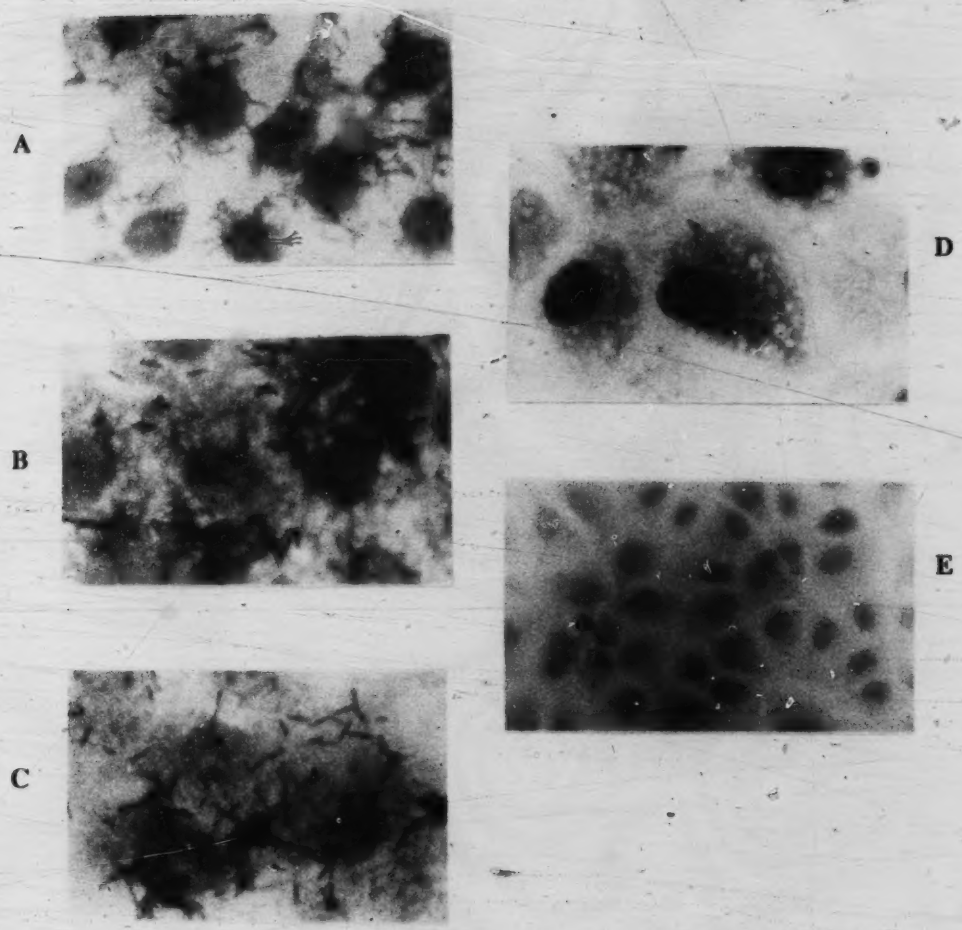


FIGURE 2. Adherence of *L. bulgaricus* strain J10 to a confluent monolayer of RI cells in different suspending media with subsequent rinsing, using different rinse solutions: A) EBSS as suspending medium, EBSS as rinse solution B) EBSS as suspending medium, EDTA as rinse solution C) EDTA + 3.6 mM Ca^{+} as suspending medium, EBSS as rinse solution The adherence of strain J8 (D) at 1000 x magnification and strain J9 (E) at 160 x magnification showing no adherence by bacterial cells.

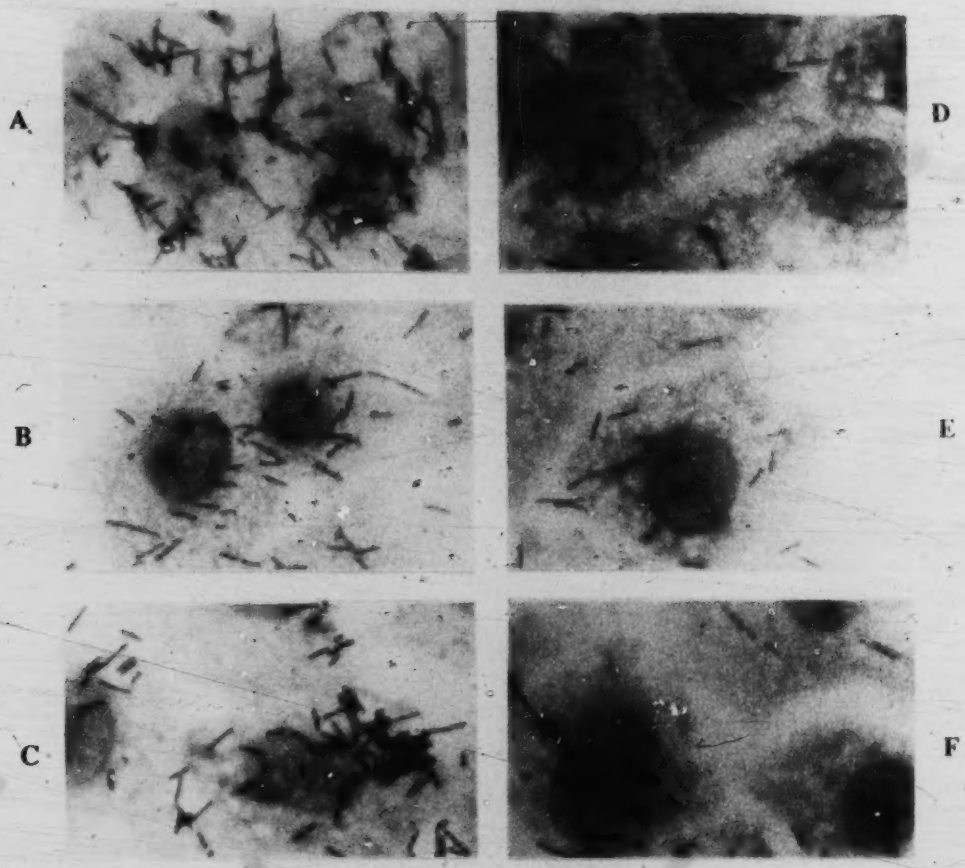


FIGURE 3A. Differences in adherence of *L. bulgaricus* strain J10 cells grown in MRS broth (A. bacterial cells suspended in EBSS and rinsed with EBSS; B. bacterial cells suspended in EBSS and rinsed with EDTA solution; C. bacterial cells suspended in EBSS + Ca ions and rinsed with EBSS), compared with cells grown in 10% RSM (D. bacterial cells suspended in EBSS and rinsed with EBSS; E. bacterial cells suspended in EBSS and rinsed with EDTA solution; F. bacterial cells suspended in EBSS + Ca ions and rinsed with EBSS).

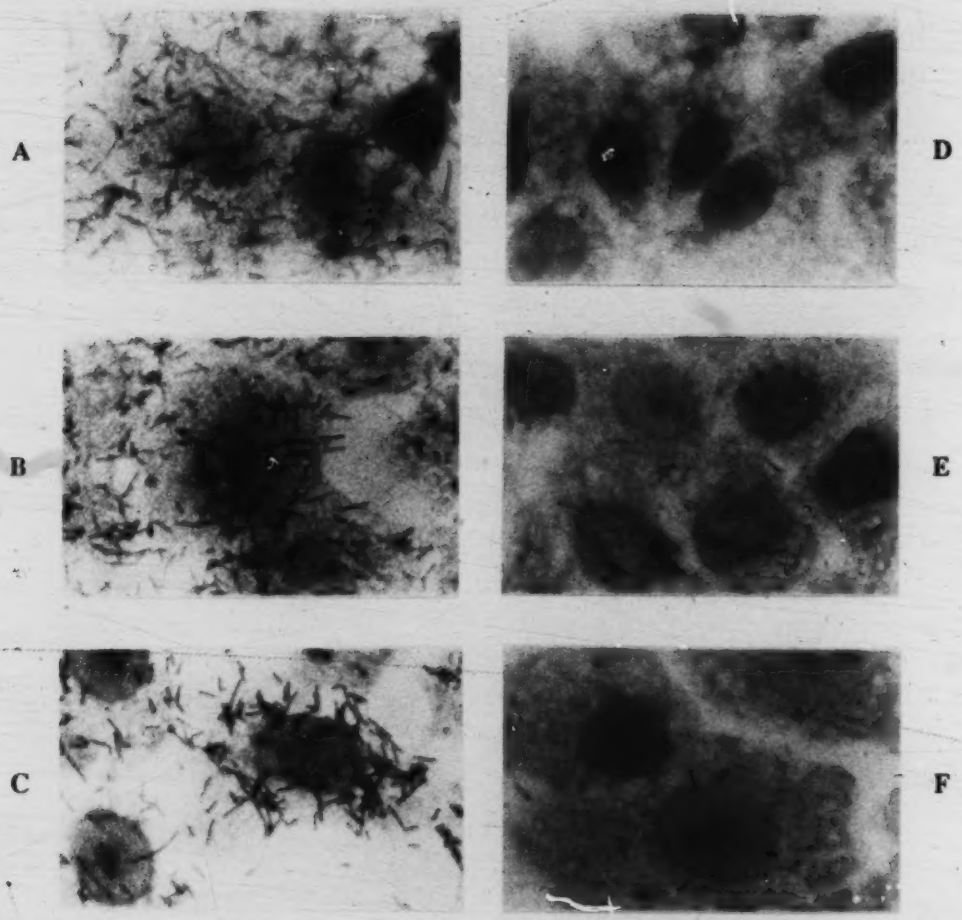


FIGURE 3B. Difference in adherence of *L. acidophilus* BG2-FO4 cells grown in MRS broth (A. bacterial cells suspended in EBSS and rinsed with EBSS; B. bacterial cells suspended in EBSS and rinsed with EDTA solution; C. bacterial cells suspended in EBSS + Ca ions and rinsed with EBSS), compared with cells grown in 10% RSM (D. bacterial cells suspended in EBSS and rinsed with EBSS; E. bacterial cells suspended in EBSS and rinsed with EDTA solution; F. bacterial cells suspended in EBSS + Ca ions and rinsed with EBSS).

scoring of the degree of adherence is shown in Tables 4A and B. Incubation temperature of bacterial cultures at 30, 37, and 40°C, did not influence the adherence of the strains tested. *L. bulgaricus* strains J8 and J9 failed to adhere to RI cells under all growth temperatures tested, while *L. bulgaricus* strains J6, J10, J11 and *L. acidophilus* strain BG2-FO4 adhered to the cells under these experimental conditions.

4.2.4 Effect of growth phase of *L. bulgaricus* strains J8 and J10 on the adherence to RI cells

Growth phases of *L. bulgaricus* strains J8 and J10 were determined in MRS cultures relative to time of incubation in MRS broth at 37°C. As shown by the growth curves in Figure 4, *L. bulgaricus* strains J8 and J10 exhibit similar growth patterns. Cells harvested at 12-14 h are in the logarithmic phase of growth; and at 20-24 h and 48 h, they are in the early and late stationary phase of growth, respectively. *L. bulgaricus* strain J8 did not adhere to RI cells; whereas *L. bulgaricus* strain J10 adhered strongly to the monolayer of RI cells when tested in the logarithmic and stationary phases of growth, regardless of the availability of calcium ions. Hence, stage of growth does not influence the adherence of *L. bulgaricus* strains J8 and J10 to RI cells.

4.2.5 Effect of chemical pretreatment of RSM cultures of *L. bulgaricus* strains J8 and J9 on adherence to RI cells

Cells of strains J8 and J9 grown in RSM were negative for adherence to RI cells. Cultures grown in RSM were pretreated with NaOH and citrate to solubilize the milk proteins in order to harvest the cells. Controls grown in MRS were tested to determine whether the chemical pretreatment affected adherence to RI cells. Controls using adhering strains, J6 and J10 grown in MRS broth, did not lose their ability to adhere to RI cells after chemical treatment with NaOH and citrate.

TABLE 4A. The effect of growth medium and incubation temperature of *L. bulgaricus* strains J6 and J10 on adherence to rat intestinal cells in vitro.

Strain	Growth Medium	Growth Temp(°C)	Suspending Medium / Rinse Solution[1]				
			EBSS/EBSS	EBSS/EDTA	-Ca/EBSS	-Ca/EDTA	+Ca/EBSS
J6	MRS	30	+	+	+	+	+
		37	+	+	+	+	+
		40	+	+	+	+	+
	RSM	37	±	±	±	±	±
J10	MRS	30	++	++	++	++	++
		37	++	+	++	++	±
		40	++	++	++	++	++
	RSM	30	+	+	+	+	+
		37	+	+	+	+	+
		40	+	+	+	+	+

1. See footnote Table 3

TABLE 4B. The effect of growth medium and incubation temperature of *L. bulgaricus* strain J11 and *L. acidophilus* BG2-FO4 on adherence to rat intestinal cells in vitro.

Strain	Growth Medium	Growth Temp(°C)	Suspending Medium / Rinse Solution[1]				
			EBSS/EBSS	EBSS/EDTA	-Ca/EBSS	-Ca/EDTA	+Ca/EBSS
J11	MRS	30	+	+	+	+	+
		37	+	+	+	+	+
		40	+	+	+	+	+
	RSM	37[2]	±	±	±	±	+
BG2-FO4	MRS	37[2]	++	++	++	++	++
	RSM	37[2]	±	±	±	±	-

1. See footnote Table 3.
2. Other temperatures not tested.

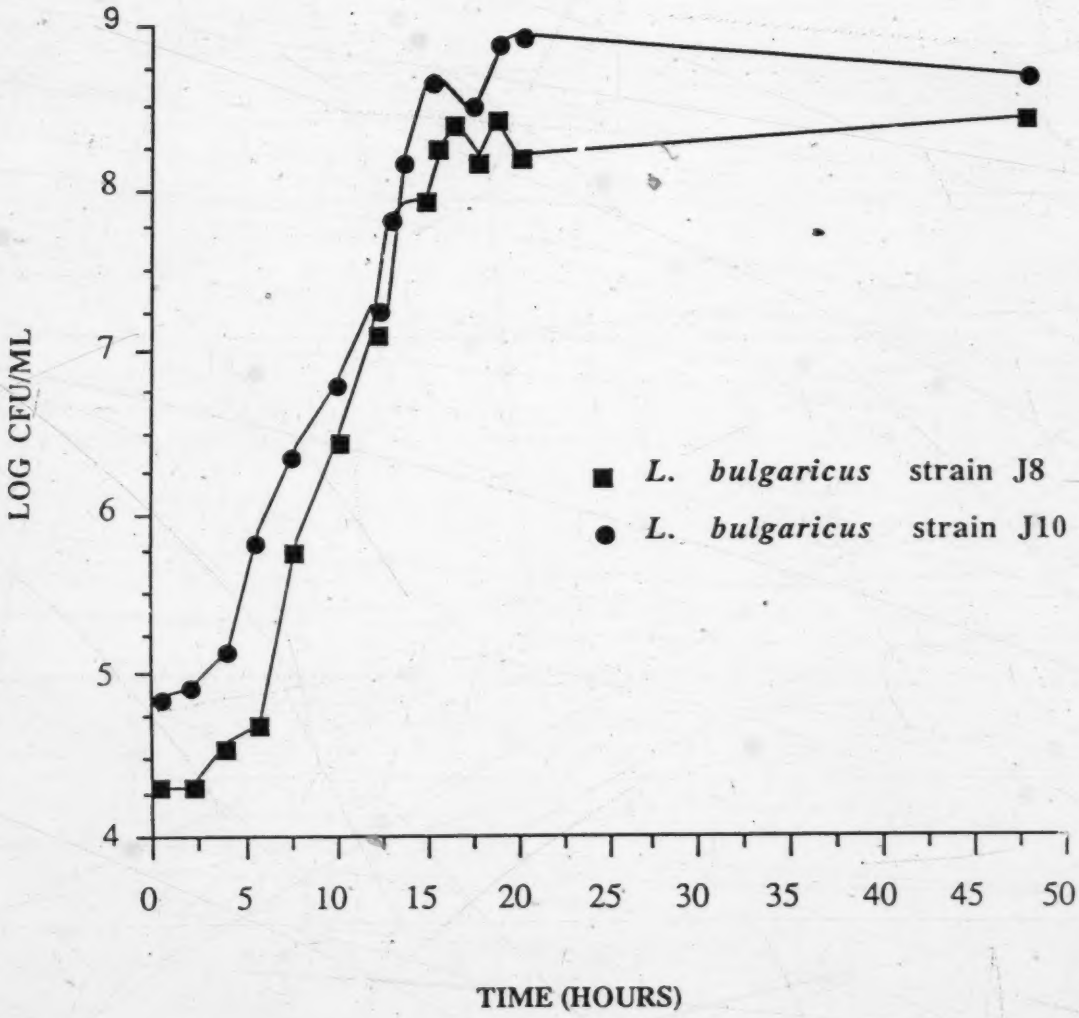


FIGURE 4. Growth curves of *L. bulgaricus* strains J8 and J10 in MRS broth at 37°C.

4.3 Characterization of *Lactobacillus* strains to develop a marker system for identification of mutants

4.3.1 Antibiograms

All *Lactobacillus* strains were tested for their resistance to the antibiotics listed in Table 5, with the intention of developing a marker system for screening mutants. All strains were resistant to polymyxin B, sulfadiazine, sulfamethoxypyridazine, colimycin and nalidixic acid up to the maximum concentration tested (256 µg/mL). In addition, *L. bulgaricus* strains were resistant to nystatin at the maximum concentration of 256 µg/mL (Table 5). From these results it was decided that polymyxin B, sulfadiazine, sulfamethoxypyridazine, colimycin and nalidixic acid should be tested for use as markers for screening mutants of test strains at 32 µg/mL concentration.

4.3.2 Production of inhibitory substances

A second possible marker system that could be developed for identifying mutants of *Lactobacillus* strains is the ability of strains to produce inhibitory substances. Inhibition was observed by the deferred method of determining antagonism (Table 6) and was in part or entirely due to the acidity of the medium or intracellular inhibitory substances in the producer strains. However, inhibition was confirmed as extracellular inhibitory substance using 18-24 h supernatant of the test strains adjusted to pH 6.5, and treated with chloroform to kill the producer strains. The inhibitory activity of the culture supernatants of producer strains J6, J10, J11 and BG2-FO4 was detected only against closely related species: *L. bulgaricus* ATCC 11842 and *L. acidophilus* ATCC 4963. Auto-inhibition was not observed when these strains were tested against themselves using both screening techniques.

The inhibitory activity observed in an inverted overlayered plate did not differ from the normal overlayered plate, indicating that inhibition was not due to the action of bacteriophage. Unlike bacteriophage, other substances such as bacteriocin in culture

TABLE 5. Highest concentration of antibiotics (µg/mL) in doubling concentrations up to 256 µg/mL tolerated by *Lactobacillus* spp.

Antibiotics	Test Strains					
	J6	J8	J9	J10	J11	BG2-FO4
Penicillin G	<1	<1	<1	<1	<1	<1
Kanamycin	16	<1	<1	<1	<1	<1
Neomycin	128	128	64	64	64	128
Gentamycin	16	<1	<1	16	16	<1
Streptomycin	16	32	4	8	16	64
Tetracycline[1]	32	32	32	32	32	32
Lincomycin	2	<1	<1	4	4	<1
Chloramphenicol	<1	<1	<1	<1	<1	<1
Rifamycin	<1	<1	<1	<1	<1	<1
Polymyxin B[2]	256	256	256	256	256	256
Sulfadiazine[2]	256	256	256	256	256	256
Sulfamethoxy-pyridazine[2]	256	256	256	256	256	256
Colimycin[2]	256	256	256	256	256	256
Nystatin	256	256	256	256	256	<1
Nalidixic Acid[2]	256	32	32	256	256	256

<1 = sensitive to the antibiotic at the lowest level tested (1 µg/mL)

1. The highest concentration tested, 32 µglmL.
2. Antibiotics used for screening mutants.

TABLE 6. Indirect screening for production of inhibitory substances by *Lactobacillus* spp. (Deferred Antagonism).

	Indicator Organisms[1]									
Test Strain	UAL9	A68	A08	A06	A37	A42	J10	J11	ADH	A63
J6	+	+	+	+	+	+	+	+	±	++
J8	+	±	±	±	+	+	±	+	-	±
J9	-	-	-	-	+	±	-	±	-	±
J10	+	+	+	+	+	+	+	+	±	++
J11	+	+	+	+	+	+	+	+	±	++
BG2-FO4	+	-	-	-	-	++	+	+	-	+

1. A68 = ATCC 23368
A08 = ATCC 4008
A06 = ATCC 12706
A37 = ATCC 1937
A42 = ATCC 11842
A63 = ATCC 4963

++ = inhibition with complete clearing and sharp edges.
+ = clearing with diffuse edges.
± = slight inhibition.
- = no inhibition.

supernatant can diffuse through the agar to inhibit the growth of indicator organisms growing on the reverse side. No difference in inhibitory activity was observed in supernatant that was treated with catalase (10% concentration), indicating that H_2O_2 was not the cause of the antagonism. Culture supernatant also lost its activity after heat treatment at 62°C for 15 min. The inhibitory activity in the supernatants of strain J10, J11 and BG2-FO4 was not affected by treatment with protease from *Streptococcus griseus* ; but treatment with Pronase^R, eliminated the inhibitory activity in the supernatants of all producer strains including strain J6, against indicator strain ATCC 4963 (Table 7). Pronase^R is a commercial preparation of a mixture of different proteases whose origins are not revealed. Inhibitory activity of strain J6 against both indicator organisms was lost after treatment with protease from *S. griseus*. These observations indicate that the inhibitory activity is due to a heat labile substance which is proteinaceous in nature. However, these tests were insufficient to show that the inhibitory substance is a bacteriocin. For the purpose of these studies however, the antagonistic substances will be referred to as bacteriocins.

4.4 Plasmid profiles of *Lactobacillus* test strains

The plasmid profiles of *Lactobacillus* strains J6, J8, J9, J10, J11 and BG2-FO4 are shown in Figure 5. The plasmid profiles of strain J6, J10 and J11 are similar, each having two extrachromosomal DNA bands at the same relative migration distance. Strains J8 and J9 appear to be plasmidless, based on these mini-scale plasmid profiles (Figure 5, second photo). *L. acidophilus* strain BG2-FO4 however has a different plasmid profile, with one large molecular size extrachromosomal DNA band traveling above the chromosomal band.

Results from two dimensional gel electrophoresis of J6, J10 and J11 show that the upper and lower band both migrated through the same distance after exposure to UV light for 15-20 min. This indicates that the upper extrachromosomal DNA band of these strains was derived from the lower extrachromosomal DNA. The gel pattern indicates that the

TABLE 7. Characteristics of inhibitory substances produced by *Lactobacillus* spp.[1]

	Indicator Organisms							
	ATCC 4963				ATCC 11842			
Strain	Control[2]	Catalase	Protease	Pronase[R]	Control[2]	Catalase	Protease	Pronase[R]
J6	++	++	±	±	+	+	-	NT[3]
	++	++	±	±	++	++	-	NT
J10	++	++	++	-	+	+	+	NT
	++	++	++	-	++	+	+	NT
J11	++	++	++	-	+	+	+	NT
	++	++	++	-	++	++	+	NT
BG2-	++	++	++	±	+	+	+	NT
FQ4	++	++	++	±	++	++	++	NT

1. See footnote Table 6.
2. Supernatant adjusted to pH 6.5 and sterilized with chloroform.
3. Not tested.

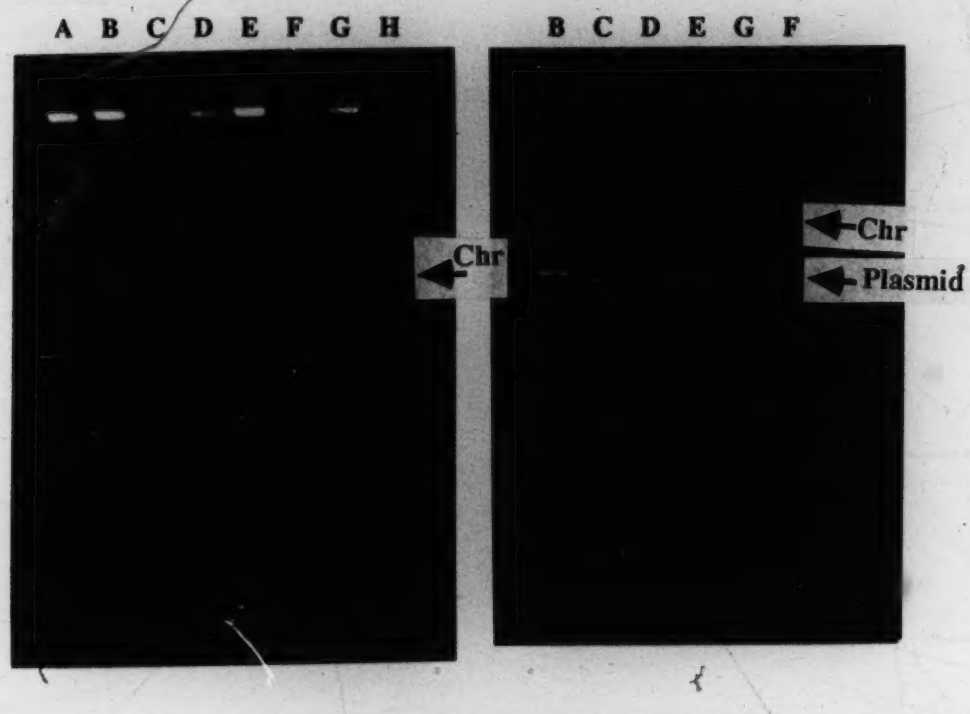


FIGURE 5. Plasmid profiles of *Lactobacillus* spp. strains J6 (B), J8 (C), J9 (D), J10 (E), J11 (F), J11-HS3 (G) and BG2-FO4 (H), with *Escherichia coli* V517 (A) as the standard.

upper band was most probably the OC form of the plasmid, hence migrating at a much slower speed than the lower band which is most probably in the CCC form. This indicates that strains J6, J10 and J11 have one plasmid each. The size of the plasmid was estimated using the plasmids from *E. coli* V517. From the plot in Figure 6 it is estimated that the size of the plasmid of strains J6, J10 and J11 is 6.3 megadalton, whereas the size of the plasmid of strain BG2-FO4 is 17.8 megadalton.

4.5 Generation of plasmidless mutants from plasmid curing

Lactobacillus strains that contained plasmids were subjected to curing reagents to obtain plasmidless mutants.

(i) The use of intercalating dyes, acriflavine and ethidium bromide, failed to generate detectable mutants. Strains exposed to curing dyes were screened for loss of bacteriocin production or for change in sensitivity to antibiotics. Plasmid isolation of potential mutant strains did not reveal plasmidless mutants.

(ii) The use of SDS in combination with novobiocin also failed to generate detectable mutants. Plasmid profiling did not reveal any plasmidless mutants.

(iii) The use of elevated temperatures in combination with novobiocin was successful in producing plasmidless mutant strains. A plasmidless mutant of *Lactobacillus* strain J11, designated J11-HS3, was identified by its plasmid profile shown in Figure 5. This mutant was identified by the appearance of its colony, which was translucent and flat as opposed to the opaque, raised colonies of the wild type. However, after a few transfers in MRS broth the colonies resumed their normal appearance. Plasmid profiling was used as a tool for the confirmation of the cured strain.

The use of elevated temperature (47-48°C) was also successful in generating plasmidless strains of *L. acidophilus* strain BG2-FO4 (Figure 7). The mutants known as FO4-1 and FO4-2 do not contain the plasmid. *L. bulgaricus* strains J6 and J10 could not be cured of their plasmids by the procedures used in this study.

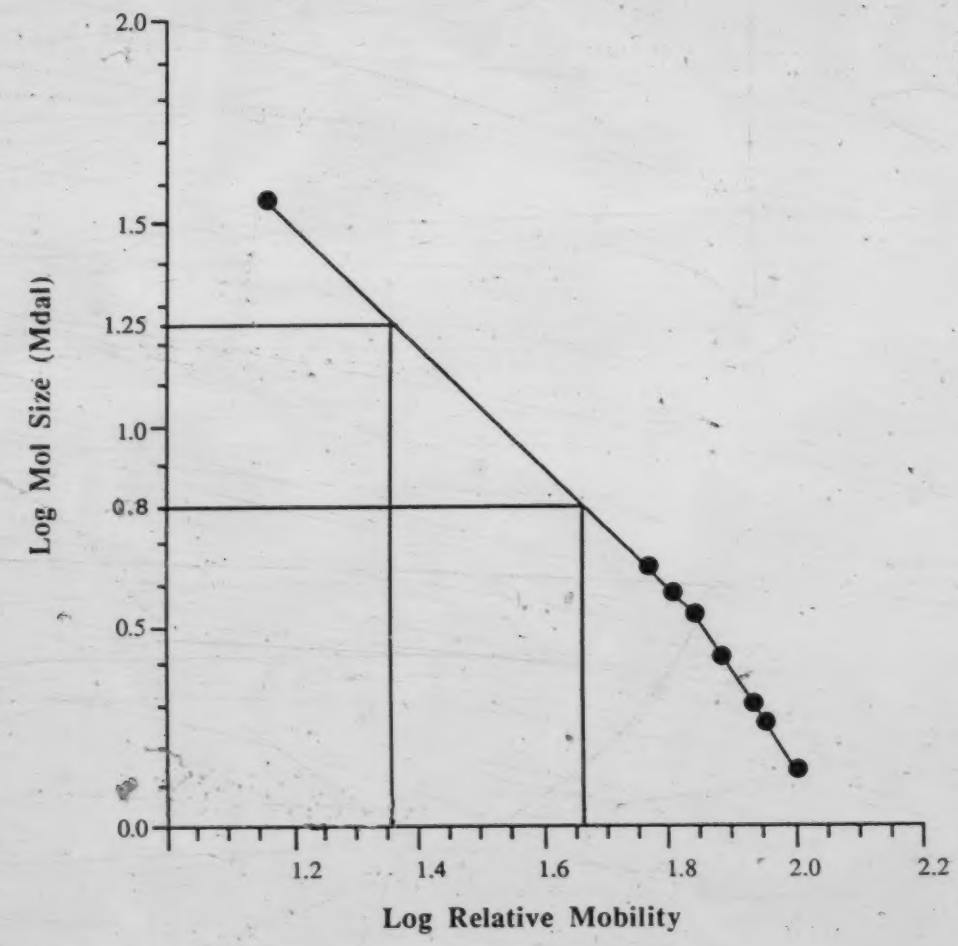


FIGURE 6. Migration of plasmids of *E. coli* V517 relative to molecular size.

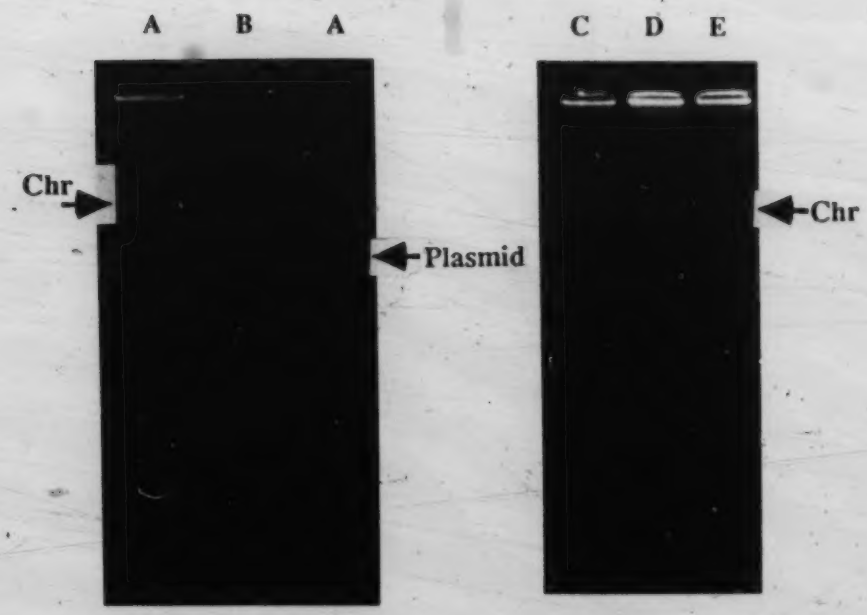


FIGURE 7. Plasmid profiles of *L. bulgaricus* strain J11 (A) and its mutant strain J11-HS3 (B), and *L. acidophilus* strain BG2-FO4 (C) and its mutant strains FO4-1 (D) and FO4-2 (E).

4.6 Selected characteristics of mutant strains

As shown in Table 8, the J11-HS3 mutant produces bacteriocin similar to the wild type. Auto-inhibition was not observed when tested against itself and against its wild type strain and also when used as the indicator for the wild type. It did not show a change in sensitivity to the antibiotics used in the screening process (See footnote Table 5). These observations indicate that the plasmid in strain J11 does not encode for bacteriocin production or resistance to antibiotics used in the screening process. Therefore they cannot be used as markers for screening mutants. Gram stain of mutant strain J11-HS3 cells did not reveal any difference in morphology from the wild type strain. In addition, it was found that carbohydrate fermentation patterns of the mutant strain J11-HS3 and the wild type strain J11 are identical. Thus carbohydrate fermentation pattern remains unchanged in the plasmidless mutant strain, and sugar fermentation cannot be used as a marker system in this study.

The inhibitory activity of mutant strain FO4-1 was similar to its wild type strain, against indicator strain ATCC 11842 and ATCC 4963. Mutant strain FO4-2, on the other hand, inhibited indicator strain ATCC 4963 but not ATCC 11842 (Table 8). Antibiotic sensitivity and carbohydrate fermentation tests were not done on these mutants.

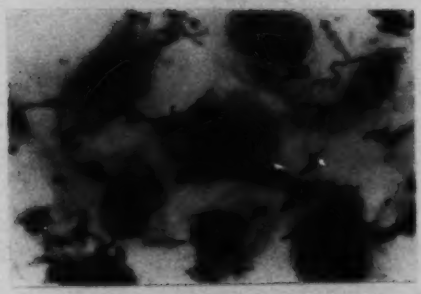
4.7 Adherence test of plasmidless mutants

The mutant strains J11-HS3, FO4-1 and FO4-2 were subjected to adherence test on RI monolayer cells. As shown in Table 8 and Figures 8A and 8B, the plasmidless mutants did not lose their ability to adhere to RI cells. This indicates that the adherence ability of *L. bulgaricus* strain J11 and *L. acidophilus*, strain BG2-FO4 is not mediated by their plasmids. At this time, these plasmids are cryptic to the apparent functioning of the cells.

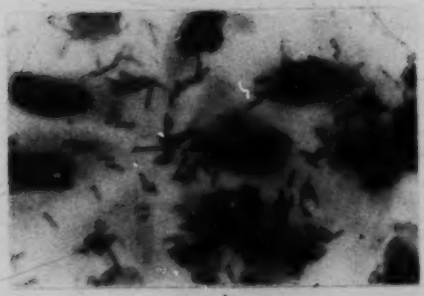
TABLE 8. Adherence and inhibitory substance produced by cured strains of *L. bulgaricus* J11 and *L. acidophilus* BG2-FO4.

	Adherence Test[1]		Inhibitory Substances[2]	
Strain of	Suspending Medium / Rinse Solution[3]		Indicator Organisms	
Lactobacillus	EBSS/EBSS	EBSS/EDTA	ATCC 11842	ATCC 4963
J11	+	+	+	+
J11-HS3	+	+	+	+
BG2-FO4	+	+	++	++
FO4-1	+	+	++	++
FO4-2	+	+	-	++

1. + = positive adherence
2. ++ = inhibition with complete clearing and sharp edges.
   + = clearing with diffuse edges.
   ± = slight inhibition.
   - = no inhibition.
3. See footnote Table 3



A



B

FIGURE 8A. The adherence of *L. bulgaricus* strain J11 (A), and its mutant strain J11-HS3 (B).

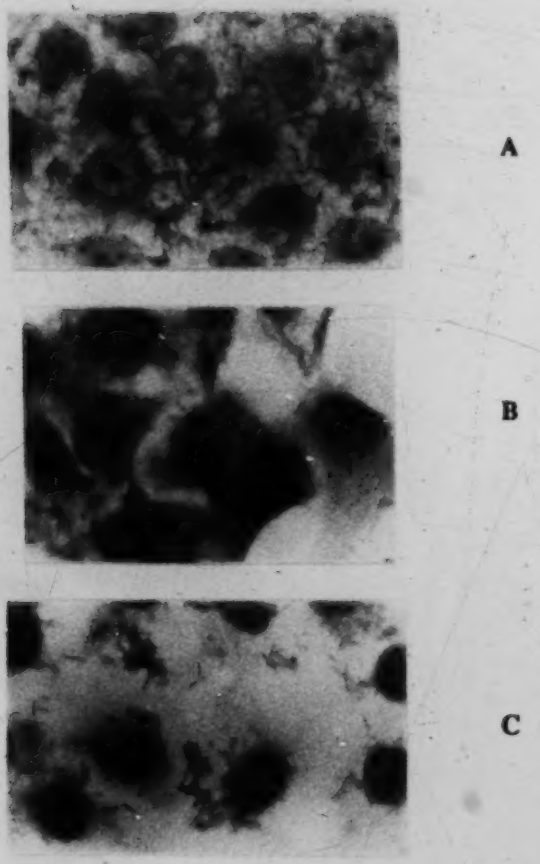


FIGURE 8B. The adherence of *L. acidophilus* strain BG2-FO4 (A), and its mutant strains FO4-1 (B), and FO4-2 (C).

5. DISCUSSION AND CONCLUSION

Lactobacillus delbrueckii subsp. *bulgaricus* (*L. bulgaricus*) is used with *Streptococcus thermophilus* for the production of yogurt. Yogurt was first associated with the prolongation of life in the early 1900's by Elie Metchnikoff (Bibel, 1988). To this day no evidence has been found to support the above claim. However, numerous studies have generated data supporting the nutritional and therapeutic use of lactobacilli in fermented foods, such as yogurt and acidophilus milk. *L. acidophilus* is frequently used by researchers for investigations related to therapeutic use while, more often than not, *L. bulgaricus* has been ignored. Several earlier reports indicated that *L. bulgaricus* does not have the ability to adhere and colonize the gut (Herter and Kendall, 1908; Rahe, 1915), while another report stated that *L. acidophilus* may be better equiped to persist in the jejunum than *L. bulgaricus* (Robins-Browne and Levine, 1981). In fact, very little is known about *L. bulgaricus* in terms of its nutritional and therapeutic use. Because yogurt is a commonly accepted food its potential benefits to consumers should be studied. Investigations of the ability of *L. bulgaricus* to adhere to intestinal cells should be done, because it is assumed that the cells must be able to adhere to the epithelial cells of the intestine if any benefits are to accrue to the individual.

The purpose of this study was to investigate the adherence of *L. bulgaricus* to rat intestinal epithelial cells. *L. acidophilus* BG2-FO4 was used as the positive control, since this strain was shown to adhere to human fetal intestinal (HFI) cells in vitro (Kleeman and Klaenhammer, 1982). Adherence testing was originally used in this study as a method for determining the viability of *L. bulgaricus* strains that exhibited marked variations in cellular morphology. *L. bulgaricus* strains J8 and J9 exhibited variations in cellular morphology when grown in MRS broth but not in reconstituted skimmilk (RSM). The phenomenon of morphological variation was reported by Britz and Dreyer (1979), but the cause of variation was not explained. They also reported the production of slime by strain J8. The

environment could be the cause of variation in cellular morphology of strains J8 and J9. The viability of these irregularly shaped cells was being questioned in its relation to their ability to adhere to intestinal epithelial cells. Hence, the effect of different growth conditions on the ability of *L. bulgaricus* strains to adhere to intestinal epithelial cells in vitro was investigated.

A rat intestinal (RI) IEC-6 cell line was used as the substrate for the in vitro adherence tests. The lack of ability of *L. bulgaricus* strains J8 and J9 to adhere to RI cells under any conditions of growth and treatment implies that they lack the adherence factor that is present in other strains. In this situation, adherence does not appear to be related to viability and the adherence test cannot be used to determine viability of cells that exhibit morphology variations. However, *L. bulgaricus* strains J6, J10 and J11 and *L. acidophilus* BG2-FO4, which do not exhibit variations in cellular morphology, were able to adhere to RI cells when grown at different incubation temperatures in MRS broth.

Fuller (1975) reported that growth medium does not affect the adherence of *Lactobacillus* spp. to epithelial cells of chicken crop. On the contrary, we observed a loss of adherence of strains J6, J10, J11 and BG2-FO4 to RI cells when grown in RSM. This could be due to the change in physiological conditions or loss of viability in RSM. However, cultures grown in RSM had to be pretreated with NaOH and citrate to facilitate harvesting. This process may have affected the cells, injuring them and (or) impairing their ability to adhere to RI cells. However, controls using cells grown in MRS with similar pretreatment with NaOH and citrate did not affect the adherence of these *Lactobacillus* strains indicating that the chemical treatment of RSM does not cause the loss of ability of strains J6, J10, J11and BG2-FO4 to adhere to RI cells. Sandine and Elliker (1970) reported that this chemical pretreatment does not affect cell activity. Hence, the loss of adherence by cells grown in RSM is related to some factor(s) affected by the growth medium.

The adherence factor in strains J6, J10, J11 and BG2-FO4 is produced when cells are grown at temperatures between 30-40°C in MRS broth, and is not affected by stage in their growth cycle. This is confirmed by Fuller (1975) who also found that incubation temperature, age of culture and active metabolism do not affect the adherence of *Lactobacillus* spp. to chicken crop epithelial cells. To the contrary, Suegara *et al.* (1975) reported that the loss of adherence to keratinized epithelial cells of the rat stomach in vitro occured in *L. fermenti* as age of culture increases. It was suggested that death of cells denatured the component that mediates attachment. However, *L. bulgaricus* strains J8 and J9 lack the factor necessary for adherence to RI cells. The concentration of cells in cultures of strains J8 and J9 bacterial suspensions was 10 times lower than that of the other bacterial suspensions adjusted to the same turbidity (see Table 2). However, this factor should not have affected the adherence of strains J8 and J9. The cell concentration could be adjusted to test this factor. Since no bacterial cells were found adhering to the monolayer of RI cells at the lower density level, adjustments in bacterial cell concentration was not necessary. Since *L. bulgaricus* strains J8 and J9 are noted to be slime producers in MRS broth and RSM from this study,the presence of slime around the bacterial cells may prevent the cells from coming in close contact with the layer of RI cells. However, staining the monolayer of RI cells with Giemsa stain did not reveal the the presence of slime layer around the cells. Britz and Dreyer (1979) observed the presence of slime by staining cell cultures with Gram's stain. However, the high viscosity of the bacterial cultures was indicative enough of the presence of slime. From this study, it is not known whether the variation in cellular morphology in strains J8 and J9 affected adherence. To investigate this, it would be necessary to find a *Lactobacillus* strain that exhibits variation in cellular morphology as well as being able to adhere to RI cells in MRS broth. Strains used in this study do not have such characteristics.

Kleeman and Klaenhammer (1982) suggested the existence of two mechanisms of adherence of *Lactobacillus* to epithelial cells. Using human fetal intestinal (HFI) cells, they

demonstrated that calcium enabled all *Lactobacillus* strains that they tested to adhere nonspecifically to HFI cells. It has been postulated that cations such as calcium act as chemical bridges for the bacterial cells to adhere to epithelial cells (Brooker and Fuller, 1975; Costerton *et al.*, 1978). In the absence of calcium ions, however, only a few strains were capable of specific adherence. In our study, calcium ions did not affect the adherence of *Lactobacillus* strains J6, J10, J11 and BG2-FO4, and did not enable strains J8 and J9 to adhere to RI cells. Hence, adherence by strains J6, J10, J11 and BG2-FO4 appears to be attributable to a specific adherence mechanism inherent to the strains. Hence, the theory of calcium ion bridge formation between bacterial and epithelial cells does not apply to *Lactobacillus* strains in our study, or else it is a factor influenced by the use of RI (IEC-6) cells in our study. *L. acidophilus* BG2-FO4 adheres to HFI cells (Kleeman and Klaenhammer, 1982) and to RI cells in our study. This illustrates that adherence of *L. acidophilus* BG2-FO4 may not be host-specific, in contrast to the report by Savage *et al.* (1968) in which it was concluded that adherence of *Lactobacillus* spp. is host-specific.

Hood and Zottola (1987) considered that adherence of *L. acidophilus* BG2-FO4 may be mediated by a polysaccharide material found on the cell wall. It was also reported that strains that showed weak adherence did not produce the polysaccharide material on the cell wall. It would be interesting to test for this material in the *Lactobacillus* strains in our study, to confirm the substance responsible for adherence. It would also be interesting to investigate if the substance responsible for adherence is affected by different growth media, especially by RSM. This would help to explain the loss of adherence of our *Lactobacillus* strains grown in RSM.

Lin and Savage (1985) suggested that adherence of *Lactobacillus* spp. to epithelial cells is mediated by plasmids. In this study, an association between adherence and genetic determinants in plasmid DNA was not observed. Preceeding this, we attempted to develop a marker system by using the production of antagonistic substances by *Lactobacillus* spp. The antagonistic substances produced by strains J10, J11 and BG2-FO4 were inactivated

by Pronase^R but not by protease from *Streptococcus griseus*. The bacteriocinogenic strains that tested positive for adherence to RI cells each had one cryptic plasmid. However, we failed to identify any function associated with the plasmids. Attempts to cure the strains of their plasmids using ethidium bromide, acriflavin and SDS in combination with novobiocin were unsuccessful. However, the use of elevated incubation temperatures, also used in combination with novobiocin, generated some mutants of strains J11, and BG2-FO4. Attempts to develop a marker system for identifying mutants of *L. bulgaricus* strain J11 were unsuccessful. Phenotypic traits, such as carbohydrate fermentation, bacteriocin production and antibiotic resistance could not be associated with the loss of plasmids. Other phenotypic traits such as resistance to metal ions and antibiotics other than the five used in this study could also be tested for possible markers. The five antibiotics used in this study were chosen because the test strains were equally and highly resistant to them.

Despite many curing trials, no mutants of *L. bulgaricus* strains J6 and J10 were generated or they could not be identified by the screening methods used in this study. *L. bulgaricus* strains J6 and J10 have identical plasmid profiles to *L. bulgaricus* strain J11, yet it is not necessarily true to state that phenotypic traits such as the ability to adhere to RI cells, bacteriocin production and immunity are genetically identical in all three strains. Failure to isolate mutants of *L. bulgaricus* strains J6 and J10 using bacteriocin production for screening may indicate that bacteriocin production and immunity are chromosomally determined in these strains. *L. acidophilus* mutant strains FO4-1 and FO4-2 were identical to the wild type strain BG2-FO4 in adherence to RI cells, indicating that adherence is chromosomally mediated in these strains. Plasmid profiles revealed that both mutants are plasmidless, however differences in bacteriocin activity were noted between mutants. Mutant strain FO4-2 lost its antagonistic activity against indicator strain ATCC 11842, while mutant strain FO4-1 exhibited similar bacteriocinogenic pattern as the wild type strain BG2-FO4. It can be postulated that two bacteriocins are produced by *L. acidophilus* BG2-FO4 which are active against different target strains. The bacteriocin active against indicator

strain ATCC 11842 was plasmid associated, however more work must be done to isolate and characterize the bacteriocin before this can be established. It may also be postulated that the plasmid for bacteriocin production and immunity in strain BG2-FO4 has been incorporated into the chromosome of strain FO4-1, thereby accounting for its antagonistic activity that is similar in pattern to its wild type. This factor can be determined by DNA hybridization on the mutant and wild type strains.

Adherence was scored subjectively based on the presence or absence of bacterial cells associated with or adhering to the RI cells. This method is subject to errors because of its subjective nature. An objective method using statistics may be an improvement. Bacterial cells attaching to epithelial cells could be enumerated in a fixed number of microscopic fields to generate data to decide if adherence was significantly different with different treatments.

This study provides evidence that chromosomal determinants may be responsible for the adherence factor, as the ability to adhere to RI cells did not change with the lost of plasmid DNA in the *Lactobacillus* spp. Efforts to associate adherence or other factors with plasmids were unsuccessful. At this point we conclude that *L. bulgaricus* strains J6, J10, J11 each contains a cryptic plasmid, while *L. acidophilus* strain BG2-FO4 contains a plasmid that may determine the production of a bacteriocin active against indicator strain ATCC 11842.

This study would have provided great potential for further research work if the adherence factor was plasmid mediated in these *Lactobacillus* strains. However, with the techniques at hand it is possible to expand this work using a wider range of *Lactobacillus* strains and a tissue culture system that is directly related to humans, such as human fetal intestinal cell line. It will be a great challenge to identify a *L. bulgaricus* strain that habours a plasmid which determines for adherence ability to intestinal epithelial cells. The plasmid can then be isolated and put into a recipient cell. *L. bulgaricus* strains that are currently being used in the production of commercial yogurt are highly desirable for use as recipient

cells. These strains already posses the characteristics that produce favorable qualities in the product and can be further enhanced by their ability to adhere to human gastrointestinal tract, so that nutritional and therapeutic benefits can be derived from their implantation. When and if such a challenge is achieved, yogurt may be used for therapeutic purposes.

6. REFERENCES

Alm, L. 1983a. The effect of *Lactobacillus acidophilus* administration upon the survival of *Salmonella* in randomly selected human carriers. Prog. Food Nutr. Sci. 7:13-17.

Alm, L. 1983b. Survival rate of *Salmonella* and *Shigella* in fermented milk products with and without added human gastric juice: An in-vitro study. Prog. Food Nutr. Sci. 7:19-28.

Amer, M. A., and A. M. Lammerding. 1983. Health maintenance benefits of cultured dairy products. Cult. Dairy Prod. J. 18:6-10.

Anderson, D. G., and L. L. McKay. 1983. Simple and rapid method for isolating large plasmid DNA from lactic streptococci. Appl. Environ. Microbiol. 46:549-552.

Ayebo, A. D., I. A. Angelo, and K. M. Shahani. 1980. Effect of ingesting *Lactobacillus acidophilus* milk upon fecal flora and enzymes activity in humans. Milchwissenschaft. 35:730-733.

Ayebo, A. D., K. M. Shahani, R. Dam, and B. A. Friend. 1982. Ion exchange separation of the antitumor component(s) of yogurt dialyzate. J. Dairy Sci. 65:2388-2390.

Barefoot, S. F., and T. R. Klaenhammer. 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl. Environ. Microbiol. 45:1808-1815.

Barefoot, S. F., and T. R. Klaenhammer. 1984. Purification and characterization of the *Lactobacillus acidophilus* bacteriocin lactacin B. Antimicrob. Agents Chemother. 26:328-334.

Barrow, P. A., B. E. Brooker, R. Fuller, and M. J. Newport. 1980. The attachment of bacteria to the gastric epithelium of the pig and its importance in the microecology of the intestine. J. Appl. Bacteriol. 48:147-154.

Beck, C., and H. Necheles. 1961. Beneficial effects of administration of *L. acidophilus* in diarrheal and other intestinal disorders. Am. Health Org. 9:1-9.

Bianchi-Salvadori, B., P. Camaschella, and E. Bazzigaluppi. 1984. Distribution and adherence of *Lactobacillus bulgaricus* in the gastroenteric tract of germ-free animals. Milchwissenschaft. 39:387-391.

Bibel, D. J. 1988. Elie Metchnikoff's bacillus of long life. ASM News. 54:661-665.

Biss, K., K. J. Ho, B. Mikkelson, L. Lewis, and C. B. Taylor. 1971. Some unique biological characteristics of the Maasai of East Africa. N. Engl. J. Med. 284:694-699.

Bogdanov, I. G., P. G. Dalev, A. I. Gurevich, M. N. Kolosov, V. P. Mal'Kova, L. A. Plemyannikova, and I. B. Sorokina. 1975. Antitumor glycopeptides from *Lactobacillus bulgaricus* cell wall. FEMS Lett. 57:259.

Bogdanov, I. G., P. Popkhristov, and R. Marinov. 1962. Anticancer effect of antibioticum bulgaricum on Sarcoma 180 and on the solid form of Ehrlich carcinoma. Abstract of the VIIIth International Cancer Congress, Moscow.

Breslaw, E. S., and D H. Kleyn. 1973. In-vitro digestibility of protein in yogurt at various stages of processing. J. Food Sci. 38:1016-1012.

Britz, T. J., J. M. Dreyer. 1979. Karakterisering van *Lactobacillus bulgaricus* stamme vir gebruik in die bereiding van joghurt (Characterization of *Lactobacillus bulgaricus* strains for use in manufacture of youhurt). S. Afri. J. Dairy Technol. 11:101-106.

Brooker, B. E., and R. Fuller. 1975. Adhesion of lactobacilli to the chicken crop epithelium. J. Ultrastruct. Res. 52:21-31.

Carlton, B. C., and B. J. Brown. 1981. Gene mutation, p. 222-242 in Manual of Methods for General Bacteriology. Gerhardt, P., R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Kreig, and G. B. Phillips (Eds.). American Society for Microbiology, Washington, DC.

Carruthers, M. M., and B. Anderson. 1979. Inhibition by polyanions of adherence by Kanagawa-positive *Vibrio parahaemolyticus:* a physicochemical effect. J. Infect. Dis. 140:119-122.

Chandan, R. C., M. A. Searles, and J. Finch. 1969a. Lipase activity of lactic cultures. J. Dairy Sci. 52:894. (Abstr.)

Chandan, R. C., P. J. Argyle, and N. Jones. 1969b. Proteolytic activity of lactic cultures. J. Dairy Sci. 52:894. (Abstr.)

Chandan, R. C., P. J. Argyle, N. Jones, and J. F. Gordon. 1970. Changes in bovine whey proteins during storage of acidified milks. Proc. XVIII Int. Dairy Congr.1E:415.

Chassy, B. M., E. M. Gibson, and A. Giuffrida. 1976. Evidence for extrachromosomal elements in *Lactobacillus*. J. Bacteriol. 127:1576-1578.

Chassy, B. M., E. M. Gibson, and A. Giuffrida. 1978. Evidence for plasmid-associated lactose metabolism in *Lactobacillus casei* subsp. *casei*. Curr. Microbiol. 1:141-144.

Clements, M. L., M. M. Levine, P. A. Ristaino, V. E. Daya, and T. P. Hughes. 1983. Exogenous lactobacilli fed to man: Their fate and ability to prevent diarrheal disease. Prog. Food Nutr. Sci. 7:29-37.

Clements, M. L., M. M. Levine, R. E. Black, R. M. Robins-Browne, L. A. Cisneros, G. L. Drusano, C. F. Lanata, and A. J. Saah. 1981. *Lactobacillus* prophylaxis for diarrhea due to enterotoxigenic *Escherichia coli*. Antimicrob. Agents Chemother. 20:104-108.

Collins, E. B. 1977. Influence of medium and temperature on end products and growth. J. Dairy Sci. 60:799-804.

Collins, E. B., and K. Aramaki. 1980. Production of hydrogen peroxide by *Lactobacillus acidophilus*. J. Dairy Sci. 63:353-357.

Conway, P. L., A. Jouper-Jaan, and S. Kjelleberg. 1985. Abstr. Annu. Meet. Am. Soc. Microbiol. 174:158. Cited in Sherman, L. A. and D. C. Savage. 1986. Lipoteichoic acids in *Lactobacillus* strains that colonize the mouse gastric epithelium. Appl. Environ. Microbiol. 52:302-304.

Conway, P. L., S. L. Gorbach, and B. R. Goldin. 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. J. Dairy Sci. 70:1-12.

Costerton, J. W., G. G. Geesey, and K. J. Cheng. 1978. How bacteria stick. Sci. Am. 238:86.

Curtis, A. S. G. 1962. Cell contact and adhesion. Biol. Rev. 37:82.

Dahiya, R. S., and M. L. Speck. 1968. Hydrogen peroxide formation by lactobacilli and its effects on *Staphylococcus aureus*. J. Dairy Sci. 51:1568-1572.

Day, M. J. 1982. Plasmids. Edward Arnold, London.

Dodds, K. L., and D. L. Collins-Thompson. 1984. Incidence of nitrite-depleting lactic acid bacteria in cured meats and in starter cultures. J. Food Prot. 47:7-10.

Dodds, K. L., and D. L. Collins-Thompson. 1985. Characteristics of reductase activity in *Lactobacillus lactis* TS4. Can. J. Microbiol. 31:558-562.

Ellen, R. P., and R. J. Gibbons. 1974. Parameters affecting adherence and tissue tropisms of *Streptococcus pyogenes*. Infect. Immun. 9:85-91.

Farmer, R. E., K. M. Shahani, and G. V. Reddy. 1975. Inhibitory effect of yogurt components upon the proliferation of ascite tumor cells. J. Dairy Sci. 58:787-788.

Fisher, K., M. C. Johnson, and B. Ray. 1985. Lactose hydrolysing enzymes in *Lactobacillus acidophilus* strains. Food Microbiol. 2:23-29.

Friend, B. A., and K. M. Shahani. 1984a. Antitumor properties of lactobacilli and dairy products fermented by lactobacilli. J. Food Prot. 47:717-723.

Friend, B. A., and K. M. Shahani. 1984b. Nutritional and therapeutic aspects of lactobacilli. J. Appl. Nutr. 36:125-153.

Friend, B. A., R. E. Farmer, and K. M. Shahani. 1982. Effect of feeding and intraperitoneal implantation of yogurt culture cells on Ehrlich ascites tumor cells. Milchwissenschaft. 37:708-710.

Fuller, R. 1973. Ecological studies on the *Lactobacillus* flora associated with the crop epithelium of the fowl. J. Appl. Bacteriol. 36:131-139.

Fuller, R. 1975. Nature of the determinant responsible for the adhesion of the lactobacilli to chicken crop epithelial cells. J. Gen. Micro. 87:245-250.

Fuller, R. 1978. Epithelial attachment and other factors controlling the colonization of the intestine of the gnotobiotic chicken by lactobacilli. J. Appl. Bacteriol. 45:389-395.

Gadelle, D., P. Raibaud, and E. Sacquet. 1985. ß-Glucuronidase activities of intestinal bacteria determined both in vitro and in vivo gnotobiotic rats. Appl. Environ. Microbiol. 49:682-685.

Gellert, M., M. H. O'Dea, T. Itoh, and J. I. Tomizawa. 1976. Novobiocin and coumermycin inhibit DNA supercoiling catalyzed by DNA gyrase. Proc. Nat. Acad. Sci. USA 73:4474-4478.

Giannella, R. A., S. A. Broitman, and N. Zamchick. 1972. Gastric acid barrier to ingested microorganisms in man: Studies in vivo and in vitro. Gut 13:251.

Gilliland, S. E., and H. S. Kim. 1984. Effect of viable starter culture bacteria in yogurt on lactose utilization in humans. J. Dairy Sci. 67:1-6.

Gilliland, S. E., and M. L. Speck. 1975. Inhibition of psychrotrophic bacteria by lactobacilli and pediococci in nonfermented refrigerated foods. J. Food Sci. 40: 903-905.

Gilliland, S. E., and M. L. Speck. 1977. Antagonistic action of *Lactobacillus acidophilus* toward intestinal and foodborne pathogens in associative cultures. J. Food Prot. 40:820-823.

Gilliland, S. E., C. R. Nelson, and C. Maxwell. 1985. Assimilation of cholesterol by *Lactobacillus acidophilus*. Appl. Environ. Microbiol. 49:377-381.

Goldin, B. R., and S. L. Gorbach. 1984. The effect of milk and *Lactobacillus* feeding on human intestinal bacterial enzyme activity. Am. J. Clin. Nutr. 39:756-761.

Goldin, B. R., L. Swenson, J. Dwyer, M. Sexton, and S. L. Gorbach. 1980. Effect of diet and *Lactobacillus acidophilus* supplements on human fecal bacterial enzymes. J. Nat. Cancer Inst. 64:255-261.

Goodenough, E. R., and D. H. Kleyn. 1976. Influence of viable yogurt microflora on digestion of lactose by rats. J. Dairy Sci. 59:601-606.

Gordon, D., W. Macrae, and D. M. Wheater. 1957. A lactobacillus preparation for use with antibiotics. Lancet. 272:899.

Grunewald, K. K. 1982. Serum cholesterol levels in rats fed skim milk fermented by *Lactobacillus acidophilus*. J. Food Sci. 47:2078-2079.

Grunewald, K. K. 1985. Influence of bacterial starter cultures on nutritional value of foods: effects of *Lactobacillus acidophilus* fermented milk on growth and serum cholesterol in laboratory animals. Cult. Dairy Prod. J. 20:26-27.

Grunewald, K. K., and L. K. Mitchell. 1983. Growth of mice fed milk fermented with *Lactobacillus acidophilus*. J. Food Prot. 46:315-317.

Hargrove, R. E., and J. A. Alford. 1978. Growth rate and feed efficiency of rats fed yogurt and other fermented milks. J. Dairy Sci. 61:11-19.

Hargrove, R. E., and J. A. Alford. 1980. Growth response of weaning rats to heated, aged, fractionated and chemically treated yogurt. J. Dairy Sci. 63:1065-1072.

Harris, R. H., and R. Mitchell. 1973. The role of polymers in microbial aggregation. Annu. Rev. Microbiol. 27:27-50.

Harrison, V. C., and G. Peat. 1975. Serum cholesterol and bowel flora in the newborn. Am. J. Clin. Nutr. 28:1351-1355.

Hartman, A. M., and L. P. Dryden. 1965. Vitamins in milk and milk products. Am. Dairy Sci. Assoc., Champaign, IL.

Hashimoto, S., K. Nomoto, T. Matsuzaki, T. Yokokura, and M. Mutai. 1984. Oxygen radical production by peritoneal macrophages and Kupffer cells elicited with *Lactobacillus casei*. Infect. Immun. 44:61-67.

Hawksworth, G., B. S. Drasar, and M. J. Hill. 1971. Intestinal bacteria and the hydrolysis of glycosidic bonds. J. Med. Microbiol. 4:451-459.

Hepner, G., R. Fried, S. St. Jeor, L. Fusetti, and R. Morin. 1979. Hypocholesteremic effect of yogurt and milk. Am. J. Clin. Nutr. 32:19-24.

Herter, C. A., A. I. Kendall. 1908. An observation on the fate of *B. bulgaricus* (in Bacillac) in the digestive tract of a monkey. J. Biol. Chem. 5:293-302.

Hintermann, G., H. M. Fischer, R. Crameri, and R. Hutter. 1981. Simple procedure for distinguishing CCC, OC, and L forms of plasmid DNA by agarose gel electrophoresis. Plasmid. 5:371-373.

Hood, S. K., and E. A. Zottola. 1987. An electron microscopic study of adherence properties of *Lactobacillus acidophilus*. J. Food Sci. 52:791-792.

Hood, S. K., and E. A. Zottola. 1988. Effect of low pH on the ability of *Lactobacillus acidophilus* to survive and adhere to intestinal cells. J. Food Sci. 53:1514-1516.

Howard, A. N. 1977. The Maasai milk and the yogurt factor: An alternative explanation. Atherosclerosis 27:383-385.

Ishiwa, H., and S. Iwata. 1980. Drug resistance plasmids in *Lactobacillus fermentum*. J. Gen. Appl. Microbiol. 26:71-74.

Izaki, K. 1981. Enzymatic reduction of mercurous and mercuric ions in *Bacillus cereus*. Can. J. Microbiol. 27:192-197.

Jasper, D. A., L. K. Massey, and L. O. Luedcke. 1984. Effect of consuming yogurts prepared with three culture strains on human serum lipoproteins. J. Food Sci. 49: 1178-1181.

Joerger, M. C., and T. R. Klaenhammer. 1986. Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. J. Bacteriol.167:439-446.

Jonnson, E., L. Bjorck, and C. O. Claesson. 1985. Survival of orally administered *Lactobacillus* strains in the gut of cannulated pigs. Livestock Prod. Sci. 12:279-285.

Kandler, O., and N. Weiss. 1986. Genus *Lactobacillus*, p.1208-1234. In Bergey's Manual of Systematic Bacteriology Vol. 2. Sneath, P. H. A., N. Mair, and M. E. Sharpe, (Eds.). Williams and Wilkins, Baltimore, MD.

Kato, I., S. Kobayashi, T. Yokokura, and M. Matai. 1981. Antitumor activity of *Lactobacillus casei* in mice. Gann 72:517-523.

Kato, I., T. Yokokura, and M. Mutai. 1983. Macrophage activation by *Lactobacillus casei* in mice. Microbiol. Immunol. 27:611-618.

Kavasnikov, E. I., and V. I. Sodenko. 1967. Antibiotic properties of *Lactobacillus brevis*. Mikrobiol. Zh. Kyviv. 29:146. (Dairy Sci. Abstr. 29:3972.)

Kent, T. H., L. J. Fischer, and R. Marr. 1972. Glucuronidase activity in intestinal contents of rats and man and relationship to bacterial flora. Proc. Soc. Exp. Biol. Med. 140:590-594.

Kilara, A., and K. M. Shahani. 1976. Lactase activity of cultured and acidified dairy products. J. Dairy Sci. 59:2031-2035.

Kiyosawa, H., C. Sugawara, N. Sugawara, and H. Miyake. 1984. Effect of skimmilk and yogurt on serum lipids and development of sudanophilic lesions in cholesterol fed rabbits. Am. J. Clin. Nutr. 40:479-484.

Klaenhammer, T. R. 1984. A general method for plasmid isolation in lactobacilli. Curr. Microbiol. 10:23-28.

Klaenhammer, T. R., and S. M. Sutherland. 1980. Detection of plasmid deoxyribonucleic acid in an isolate of *Lactobacillus acidophillus*. Appl. Environ. Microbiol. 35:592-600.

Kleeman, E. G. and T. R. Klaenhammer. 1982. Adherence of *Lactobacillus* species to human fetal intestinal cells. J. Dairy Sci. 65:2063-2069.

Kohwi, Y., K. Imai, Z. Tamura, and Y. Hashimoto. 1978. Antitumor effect of *Bifidobacterium infantis* in mice. Gann 69:613-618.

Kohwi, Y., Y. Hashimoto, and Z. Tamura. 1982. Antitumor and immunological adjuvant effect of *Bifidobacterium infantis* in mice. Bifidobact. Microfl. 1:61.

Kondo, J. K., and L. L. McKay. 1985. Gene transfer systems and molecular cloning in group N streptococci. A review. J. Dairy Sci. 68:2143-2159.

Kotarski, S. F., and D. C. Savage. 1979. Models for study of the specificity by which indigenous *Lactobacillus* adhere to murine gastric epithelia. Infect. Immun. 26:966-975.

Kwan, A. J. 1977. Comparative B-vitamin content and organoleptic qualities of cultured and acidified sour cream. M. S. Thesis, Univ. of Nebraska-Lincoln.

Lin, J. H., and D. C. Savage. 1985. Cryptic plasmids in *Lactobacillus* strains isolated from the murine gastrointestinal tract. Appl. Environ. Microbiol. 49:1004-1006.

Macrina, F. L., D. J. Kopecko, K. R. Jones, D. J. Ayers, and S. M. McCowen. 1978. A multiple plasmid-containing *Escherichia coli* strain: Convenient source of size reference plasmid molecules. Plasmid 1:417-420.

Maffei, H. V. L., and F. J. Norbrega. 1975. Gastric pH and microflora of normal and diarrhetic infants. Gut 16:719-726.

Mann, G. V. 1977a. A factor in yogurt that lowers serum cholesterol in man. Artherosclerosis 26:335-340.

Mann, G. V. 1977b. Hypocholesteremic effect of milk. Lancet 2:556.

Mann, G. V., and A. Spoerry. 1974. Studies on a surfactant and cholesteremia in the Maasai. Am. J. Clin. Nutr. 27:464-469.

Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. 3:208-218.

Marshall, K. C. 1976. Interfaces in microbial ecology. Harvard University Press, Cambridge, Mass. Cited in Kotarski, S., and D. C. Savage. Models for study for the specificity by which indigenous lactobacilli adhere to murine gastric epithelia. Infect. Immun. 26:966-975.

Massey, L. K. 1984. Effect of changing milk and yogurt consumption on human nutrient intake and serum lipoproteins. J. Dairy Sci. 67:255-262.

Mattick, A. T. R., and A. Hirsch. 1949. The streptococci and antibiotics. XIIth Int. Dairy Congr. Papers and Commun. 546:2-11.

McCormick, E. L., D. Savage. 1983. Characterization of *Lactobacillus* sp. strain 100-37 from the murine gastrointestinal tract: ecology, plasmid content, and antagonistic activity toward *Clostridium ramosum* H1. Appl. Environ. Microbiol. 46:1103-1112.

Mikolajcik, E. M., and I. Y. Hamdan. 1975. *Lactobacillus acidophilus* II Antimicrobial agents. Cult. Dairy Prod. J. 10:18.

Morelli, L., M. Vescovo, and V. Bottazzi. 1983. Plasmids and antibiotic resistances in *Lactobacillus helveticus* and *Lactobacillus bulgaricus* isolated from natural whey culture. Microbiologica 6:145-154.

Morelli, L., M. Vescovo, P. Cocconcelli, and V. Bottazzi. 1986. Fast and slow milk coagulating variants of *Lactobacillus helveticus* HLM-1. Can. J. Microbiol. 32:758-760.

Moscoso, W., A. M. Joe, and K. M. Shahani. 1973. Comparative B-complex vitamin content of cottage cheese made by culture and direct acidification methods. J. Dairy Sci. 56:626. (Abstr.)

Muriana, P. M., and T. R. Klaenhammer. 1987. Conjugal transfer of plasmid-encoded determinants for bacteriocin production and immunity in *Lactobacillus acidophilus* 88. Appl. Environ. Microbiol. 53:553-560.

Nieman, C. 1954. Influence of trace amounts of fatty acids on the growth of microorganisms. Bacteriol. Rev. 18:147-163.

Nilson, K. M., J. R. Vakil, and K. M. Shahani. 1965. B-complex vitamin content of cheddar cheese. J. Nutr. 86:362-368.

Niv, M., W. Levy, and N. M. Greenstein. 1963. Yogurt in the treatment of infantile diarrhea. Clin. Pediatr. 2:407-411.

Pearce, J. L., and J. R. Hamilton. 1974. Controlled trial of orally administered lactobacilli in infant diarrhea. J. Pediatr. 84:261-262.

Perdigon, G., M. E. N. Macias, S. Alvarez, G. Oliver, and A. A. P. De Ruiz Holgado. 1986. Effect of perorally administered lactobacilli on macrophage activation in mice. Infect. Immun. 53:404-410.

Petterson, L., W. Graf, L. Alm, S. Lindwall, and A. Stromberg. 1983. Survival of *Lactobacillus acidophilus* NCDO 1748 in the human gastrointestinal tract. I. Incubation with gastric juice in vitro. Sympo Swed. Nutr. Foun. XV p.123. Cited in Hood, S. K., and E. A. Zottola. Effect of low pH on the ability of *Lactobacillus acidophilus* to survive and adhere to human intestinal cells. J. Food Sci. 53:1514-1516.

Plotkin, B., and D. Bemis. 1980. Evidence for two mechanisms of adherence in *Bordetella bronchioseptica* . Abstract from Annual Meeting of the American Society for Microbiology 1980 p.26.

Poupard, J. A., I. Husain, and R. F. Norris. 1973. Biology of Bifidobacteria. Bacteriol. Rev. 37:136-165.

Poznanski, S., J. Lenoir, and G. Mocquot. 1965. The proteolysis of casein under the action of certain bacterial endoenzymes. Le Lait 45:3-26.

Prajapati, J., R. K. Shah, and J. M. Dave. 1986. Nutritional and therapeutic benefits of a blended-spray dried acidophilus preparation. Cult. Dairy Prod. J. 21:16-17, 20-21.

Price, R. J., and J. S. Lee. 1970. Inhibition of *Pseudomonas* species by hydrogen peroxide producing lactobacilli. J. Milk Food Technol. 33:13-18.

Pulusani, S. R., and D. R. Rao. 1983. Whole body, liver and plasma cholesterol levels in rats fed thermophilus, bulgaricus and acidophilus milks. J. Food Sci. 48: 280-281.

Quaroni, A., J. Wands, R. L. Trelstad, and K. J. Isselbacher. 1979. Epitheliod cell cultures from rat small intestine. Characterization by morphologic and immunologic criteria. J. Cell Biol. 80:248-265.

Rahe, A. H. 1915. A study of the so-called implantation of *Bacillus bulgaricus*. J. Infect. Diseases 16:210-220.

Rao, D. R., C. B. Chawan, and S. R. Pulusani. 1981. Influence of milk and thermophilus milk on serum cholesterol levels and hepatic cholesterogenesis in rats. J. Food Sci. 46:1339-1341.

Rasic, J. L., and J. A. Kurmann. 1978. Fermented fresh milk products, Vol I. p. 120-136. Technical Dairy Publishing House, Copenhagen, Denmark.

Rasic, J., T. Stojsavljevic, and R. Curcic. 1971. A study of the amino acids of yogurt. II. Amino acids content and biological value of protein of different kinds of yogurt. Milchwissenschaft 26:219-224.

Reddy, K. P. 1972. Comparative B-vitamin content of cultured and acidified yogurt. M.S. Thesis, Univ. of Nebraska-Lincoln.

Reddy, K. P., K. M. Shahani, and S. M. Kulkarni. 1976. B-complex vitamins in cultured and acidified yogurt. J. Dairy Sci. 59:191-195.

Reddy, K. P.., K. M. Shahani, B. A. Friend, and R. C. Chandan. 1984. Natural antibiotic activity of *Lactobacillus acidophilus* and *bulgaricus*. III: Production and partial purification of bulgarican from *Lactobacillus bulgaricus*. Cult. Dairy Prod. J. 19:7-11.

Reif, G. D., K. M. Shahani, J. R. Vakil, and L. K. Crowe. 1976. Factors affecting the B-complex vitamin content of cottage cheese. J. Dairy Sci. 59:410-415.

Richardson, T. 1978. The hypocholesterolemic effect of milk. A review. J. Food Prot. 41:226-235.

Robins-Browne, R. M, and M. M. Levine. 1981. The fate of ingested lactobacilli in the proximal small intestine. Am. J. Clin. Nutr. 34:514-519.

Rochelle, P. A., J. C. Fry, M. J. Day, and M. J. Bale. 1985. An accurate method for estimating the sizes of small and large plasmids and DNA fragments by gel electrophoresis. J. Gen. Microbiol. 132:53-59.

Rossouw, J. E., E. M. Burger, P. V. D. Vyver, and J. J. Ferriera. 1981. The effect of skimmilk, yogurt, and full cream milk on human serum lipids. Am. J. Clin. Nutr. 34:351-356.

Sandine, W. E. 1979. Roles of the *Lactobacillus* in the intestinal tract. J. Food Prot. 42:259-262.

Sandine, W. E., and P. K. Elliker. 1970. Preparation of concentrates of lactic streptococci from milk cultures. J. Dairy Sci. 53:637 (Abstr.)

Sandine, W. E., K. S. Muralidhara, P. R. Eliker, and D. C. England. 1972. Lactic acid bacteria in food and health: A review with special reference to enteropathogenic

E. coli as well as certain enteric diseases and their treatment with antibiotics and lactobacilli. J. Milk Food Technol. 35:691-702.

Savage, D. C. 1969. Microbial interference between indigenous yeast and lactobacilli in the rodent stomach. J. Bacteriol. 98:1278-1283.

Savage, D. C. 1972. Association and physiological interaction of indigenous microorganisms and gastrointestinal epithelia. Am. J. Clin. Nutr. 25:1372-1379.

Savage, D. C. 1979. Introduction to mechanisms of association of indigenous microbes. Am. J. Clin. Nutr. 32:113-118.

Savage, D. C. 1983. Mechanisms by which indigenous microorganisms colonize gastrointestinal epithelial surfaces. Prog. Food Nutr. Sci. 7:65-74.

Savage, D. C., R. Dubos, and R. W. Schaedler. 1968. The gastrointestinal epithelium and its autochthonous bacterial flora. J. Exp. Med. 127:67-76.

Schleifer, K. H. 1987. Recent changes in the taxonomy of lactic acid bacteria. FEMS Microbiol. Rev. 46:201-203.

Shahani, K. M., and R. C. Chandan. 1979. Nutritional and healthful aspects of cultured and culture-containing dairy foods. J. Dairy Sci. 62:1685-1694.

Shahani, K. M., J. R. Vakil, and A. Kilara. 1976. Natural antibiotic activity of *Lactobacillus acidophilus* and *bulgaricus* I: Cultural conditions for the production of antibiosis. Cult. Dairy Prod. J. 11:14-17.

Shahani, K. M., J. R. Vakil, and A. Kilara. 1977. Natural antibiotic activity of *Lactobacillus acidophilus* and *bulgaricus*. II: Isolation of acidophilin from *L. acidophilus*. Cult. Dairy Prod. J. 12:8-11.

Shaw, B. G., and C. D. Harding. 1985. Atypical lactobacilli from vacuum-package meats: comparison by DNA hybridization, cell composition and biochemical tests with a description of *Lactobacillus carnis* sp. n. v. System Appl. Microbiol. 6:291-297.

Sherman, L. A., and D. C. Savage. 1986. Lipoteichoic acids in *Lactobacillus* strains that colonize the mouse gastric epithelium. Appl. Environ. Microbiol. 52:302-304.

Simhaee, E., and K. Keshavarz. 1974. Comparison of gross protein value and metabolizable energy of dried skim milk and dried yogurt. Poult. Sci. 53:184-191.

Sinha, D. K. 1979. Development of a nonfermented acidophilus milk and testing its properties. MS thesis, University of Nebraska-Lincoln, U.S.A.

Siver, R. H. 1961. *Lactobacillus* for the control of acne. J. Med. Soc. New Jersey. 58:52.

Smiley, M. B., and V. Fryden. 1978. Plasmids, lactic acid production, and *N*-acetyl-D-glucosamine fermentation in *Lactobacillus helveticus* subsp. *jugurti*. Appl. Environ. Microbiol. 35:777-781.

Speck, M. L. 1976. Interactions among lactobacilli and man. J. Dairy Sci. 59:338-343.

Speck, M. L. 1980. Preparation of lactobacilli for dietary uses. J. Food Prot. 42:65-67.

Stanisich, V. A. 1984. Identification and analysis of plasmids at the genetic level. p. 5-32. In Methods in Microbiology, Vol. 17. Bennett, P. M., and J. Grinsted, (Eds.). Academic Press, New York.

Suegara, N., J. E. Siegal, and D. C. Savage. 1979. Ecological determinants in microbial colonization of the murine gastrointestinal tract: Adherence of *Torulopsis pintopesii* to the epithelial surfaces. Infect. Immun. 25:139-145.

Suegara, N., M. Morotomi, T. Watanabe, Y. Kawai, and M. Mutai. 1975. Behavior of microflora in the rat stomach: Adhesion of lactobacilli to the keratinized epithelial cells of the rat stomach in vitro. Infect. Immun. 12:173-179.

Tagg, J. R. , A. S. Dajani, and W. Wannamaker. 1976. Bacteriocins of gram-positive bacteria. Bacteriol. Rev. 40:722-756.

Tamine, A. Y., and H. C. Deeth. 1980. Yogurt: Technology and Biochemistry. J. Food Prot. 43:939-977.

Thakur, C. P., and A. N. Jha. 1981. Influence of milk, yogurt and calcium on cholesterol-induced atherosclerosis in rabbits. Atherosclerosis 39:211-215.

Thompson. L. U., D. J. A. Jenkins, M. A. Amer, R. Reichert, A. Jenkins, and J. Kamulsky. 1982. The effect of fermented and unfermented milks on serum cholesterol. Am. J. Clin. Nutr. 36:1106-1111.

Tomic-Karovic, K., and J. J. Fanjek. 1962. Acidophilus milk in therapy of infantile diarrhea caused by pathogenic *E.coli*. Ann. Pediatr. 199:625-634.

Tortuero. G., A. Brene, and J. Tioperez. 1975. The influence of intestinal (cecal) flora on serum cholesterol. Am. J. Clin. Nutr. 36:1106 -1111.

Trevors, J. T. 1986. Plasmid curing in bacteria. FEMS Microbiol. Rev. 32:149-157.

Twinning-McMath, W. F. 1959. Modern trends in treatment of acute infectious diseases. Brit. J. Clin. Pathol. 13:175-181.

Vescovo, M., L. Morelli, and V. Bottazzi. 1982. Drug resistance plasmids in *Lactobacillus acidophilus* and *Lactobacillus reuteri*. Appl. Environ. Microbiol. 43:50-56.

Vincent, J. G., R. C. Veomett, and R. I. Riley. 1959. Antibacterial activity associated with *Lactobacillus acidophilus*. J. Bacteriol. 78:477-484.

Watkins, B. A., and B. F. Miller. 1983. Colonization of *Lactobacillus acidophilus* in the gnotobiotic chicks. Poult. Sci. 62:2152-2157.

Watkins, B. A., B. F. Miller, and D. H. Neil. 1982. In vivo inhibitory effects of *Lactobacillus acidophilus* against pathogenic *Escherichia coli* in gnotobiotic chicks. Poult. Sci. 61:1298-1308.

Wheater, D. M., A. Hirsch, and A. T. R. Mattick. 1952. Possible identity of *Lactobacillus* with hydrogen peroxide produced by lactobacilli. Nature 170:623-624.

Yakult Honsha Co., Ltd. 1971. The summary of reports on Yakult. Cited in Shahani, K. M., and A. D. Ayebo. 1980. Role of dietary lactobacilli in gastrointestinal microecology. Am. J. Clin. Nutr. 33:2448-2457.

Zychowicz, C., A. Surazynska, B. Siewerska, T. Cieplinska. 1974. The effect of bacterial cultures of *Lactobacillus acidophilus* (acidophilic milk) on carrier state of *Shigella* and *Salmonella* organisms in children. Pediatr. Polska. 8:997-1002.